PROTEINS OF THE SYMPATHETIC NERVOUS SYSTEM

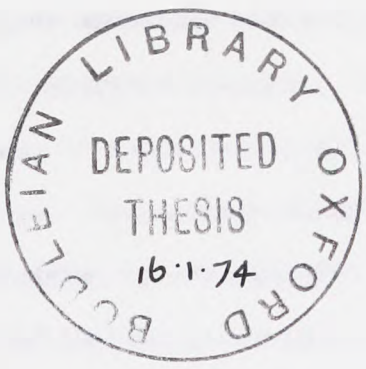
Abstract of a thesis submitted for the degree of D. Phil.

by

S. F. BARTLETT

CHRIST CHURCH

October, 1973.



ABSTRACT.

This thesis describes studies of the membrane proteins of bovine adrenal chromaffin granules (the catecholamine-storing particles of chromaffin cells), of the proteins of the related "large dense-cored vesicles" of bovine splenic nerve axons and of protein synthesis in rat superior cervical ganglia.

1. Chromaffin Granule Proteins.

Electrophoretic Experiments. After initial experience with the electrophoretic system described by previous investigators for the analysis of chromaffin granule membrane proteins (which utilises 1% SDS in borate-buffered 3.5% polyacrylamide gels, pH 9.6) an improved electrophoretic system was developed and used for molecular weight studies. The improved method involved electrophoresis in 6% gels containing 0.1% SDS and 0.1M sodium phosphate buffer pH 6.5, followed by fixation in 25% trichloroacetic acid, before staining.

This method was used to determine the electrophoretic behaviour of unreduced chromaffin granule membrane proteins, the majority of which were found to have anomalous mobilities which did not follow the predictions of the Ferguson equation. The anomalous proteins were shown, by staining by the periodic acid/Schiff reagent method, to contain carbohydrate. One protein which contained no carbohydrate and which exhibited regular electrophoretic properties was chromomembrin B, one of the two major membrane proteins. This protein was estimated to have a molecular weight of 28,500 from

measurements in 6% polyacrylamide gels. It was unaffected by treatment with dithiothreitol.

Quantitative densitometry was used to try to determine the degree of contamination of the membrane preparation with residual soluble proteins. The major soluble protein, chromogranin A, was isolated in a highly-purified form by gel-filtration and ion-exchange chromatography and used to calibrate the densitometer. It was found that ca. 13% of the protein in an SDS-extract of chromaffin granule membranes was chromogranin A and this result was discussed in the light of various conflicting literature claims.

Ion-exchange chromatography was also used to prepare a homogeneous sample of soluble dopamine β-hydroxylase. This sample was reduced and run in the phosphate/SDS gel system, when it was found to give only one stained band. It was suggested that earlier reports that this enzyme gives two sub-units on reduction were erroneous and that the "second" "sub-unit" was probably a contaminating protein. This experiment gave a molecular weight of ca. 73,500 for fully-reduced dopamine β-hydroxylase and it was suggested that the native enzyme (which has a molecular weight of ca. 290,000) was probably tetrameric. The electrophoretic behaviour of the SDS-denatured, unreduced form of the enzyme was discussed on this basis and it was suggested that this form might show considerable residual molecular assymetry, resulting in a false value for the molecular weight if the determination was carried out in gels in the absence

of reducing agents.

Gel-filtration Experiments. With the improved resolution afforded by the phosphate/SDS electrophoretic system, it was found that the Sephadex G-200 column method described for the purification of the two major membrane proteins did not yield completely homogeneous samples, and a series of experiments were carried out to establish the optimum bed material for gel-filtration separation of the proteins. The best separation was achieved with Sepharose 6B resin in a 90cm x $8cm^2$ column and this system was used preparatively to make milligramme quantities of chromomembrin A and chromomembrin B. Even with this improved gel-filtration procedure, however, a completely pure preparation could not be obtained in one chromatographic step and the partly-purified proteins were each chromatographed for a second time on the same column, after ultrafiltration to a suitable volume. Electrophoretic analysis showed that, even after this step, none of the proteins had been isolated in a pure state, and it was concluded that earlier claims in the literature that this end had been achieved with a single separation on Sephadex G-200 were open to doubt. Additionally, it was found that one of the characteristic proteins of the granule membrane - the cytochrome - was degraded during this prolonged isolation process, with the loss of its identifying spectral properties.

Molecular weight values obtained by calibrating the large column with unreduced standard proteins were found to agree rather

well with those obtained by electrophoresis, unreduced chromomembrin A once again showing an apparent molecular weight of ca. 135,500, which is not obviously related either to the molecular weight of the native enzyme or to that of the fully-reduced sub-unit.

2. Splenic Nerve Vesicle Proteins.

Samples enriched with large dense-cored vesicles, prepared from bovine splenic nerve axons in the Karolinska Institute and transported to Oxford, were osmotically shocked with tris-sodium succinate buffer (0.005M, pH 5.9) and the soluble proteins were examined, using the tris-glycine electrophoretic buffer system. The soluble proteins from the fractions throught to be enriched with nerve vesicles showed a stained band with the mobility of dopamine β-hydroxylase, one of the proteins believed to be located in these vesicles, but only a very faint band was observed in the position expected of chromogranin A. This observation was confirmed using repurified fractions and in these fractions it was particularly noticeable that the dopamine β-hydroxylase band appeared to be stronger in the less dense of the two gradient-fractions (RII). This observation was related to the possibility of vesicle damage during the isolation process. The faintness of the chromogranin A band in these samples was at first puzzling, but this was explained by an earlier report in which it was found that splenic nerve vesicles contained about 60 times as much dopamine β-hydroxylase, when expressed in terms of the noradrenaline content, as adrenal

medullary chromaffin granules (whose soluble proteins show a prominent chromogranin A band).

When the insoluble proteins of the splenic nerve vesicle fractions were solubilised with SDS and analysed, a much more complex pattern was observed than is found for the membrane proteins of chromaffin granules. Two possible explanations could be envisaged for this observation: either the granule membrane of the splenic nerve vesicle is much more complex, in terms of protein composition, than that of the chromaffin granule, or the preparations were quite heavily contaminated by proteins of non-vesicular origin. The latter explanation may be indicated by Lagercrantz's own biochemical studies, which indicated a purity of ca. 18%, and this is supported by the fact that one of the most prominent bands in the electrophoretic patterns of both soluble and insoluble proteins had a mobility close to that expected for bovine serum albumin.

3. Protein Synthesis in Rat Superior Cervical Ganglion.

Use was made of the separative capacity of the phosphate/SDS/6% polyacrylamide gel electrophoretic system to study newly-synthesised proteins in rat superior cervical ganglia incubated with radioactive leucine. After electrophoretic separation of the proteins, gels were chopped into 1.0mm slices with a sectioning-apparatus and extracted with Soluene 100 tissue solubiliser, to release the protein-bound radioactivity, which was estimated by scintillation-counting. Since published details of the overall reliability of

the method were meagre, some emphasis was placed on the evaluation of the possible sources of error in each of the stages in the analysis leading to the construction of the "labelling-profile". The pipetting, electrophoresis and scintillation-counting steps were found not to introduce significant errors into the profiles, or to distort recoveries appreciably, and the least reliable stage in the analysis was found to be the mechanical sectioning of the frozen gel, which may give rise to both random and systematic errors. While the random errors arising from variations in section-thickness could be estimated as being about 5%, the systematic error was inherent in the method of sectioning and could not be determined. This error arises from the fact that the gel cannot be accurately aligned with the razor-blade at the commencement of sectioning and from more subtle effects which follow from trying to represent a continuous distribution by a discontinuous one. A computer-simulation model of the sectioning process was developed which indicated the way in which these more subtle errors might distort the labelling-pattern under those circumstances in which the slice-thickness approaches the inter-peak spacing and the peak width. Although reducing the section thickness would be expected to reduce this kind of sectioning error, it was shown experimentally that a reduction in the slice thickness from 1.0 to 0.6mm did not have much effect on the observed resolution, whereas it lowered the accuracy with which the dpm in each slice could be determined and

much increased the labour of the experiment.

The other main source of variation between experiments lay in the efficiency of the Soluene-extraction process used to release the protein-bound radioactivity into the bulk phase, where it could be counted. Different batches of Soluene were found to give widely-differing recoveries of radioactivity and, since it was necessary to elute the radioactive label from the gel matrix before it could be counted even when using ^{14}C-leucine, it would seem likely that the variation in the recoveries was caused by variable extraction-efficiency. It was, however, found that the reduced extraction did not, in general, lead to some proteins being extracted preferentially but, instead, caused a general depression of the whole profile.

A number of experiments with both ^{14}C- and ^{3}H-leucine served to show that the incorporation of label by normal resting ganglia gave rise to a characteristic double-humped labelling-profile which was more or less evident according to the quantity of radioactivity applied to the gel, high loadings giving patterns which were rather exaggerated although still showing peaks at the same relative mobility values. A reduction in the labelling-period from 2.5 hours to 20 min did not lead to much increase in the selectivity of labelling, as judged from the observed pattern, but necessitated the use of ^{3}H-leucine of high specific activity, with associated problems in the scintillation-counting. Experiments involving stimulation of the ganglia, possibly together with chronic denervation,

failed to reveal any marked alteration in the labelling-pattern in response to changes in the environment of the ganglia and this lack of effect was discussed in the light of possible changes in the size of the intraganglionic pool of leucine and its specific activity. In spite of the relative lack of apparent control of the rate of synthesis by environmental factors, it was thought likely that the labelling-profile does in fact represent newly-synthesised secretory (soluble) proteins.

PROTEINS OF THE SYMPATHETIC NERVOUS SYSTEM.

Thesis submitted for the degree of D. Phil.

by

S. F. BARTLETT

CHRIST CHURCH

October, 1973

ACKNOWLEDGEMENTS.

To its creator, a thesis represents more than just a catalogue of scientific endeavour; it is inevitably the product of circumstance and as much a record of friendships and influences as of experimental observations. While it is invidious to apportion praise and blame, it is no mere formality, but instead a pleasure, to acknowledge how much I owe to my supervisor Dr. A.D. Smith for his interest, encouragement and friendship throughout the last three years. I am also grateful to Professor W.D.M. Paton for the privilege of working in his Department.

I should like to thank Dr. H. Lagercrantz of the Department of Physiology I, Karolinska Institutet, Stockholm for providing materials for studies described in chapter 5, and I thank Dr. Paola Paggi and Professor Giovanni Toschi of the Institute of General Physiology, University of Rome for their collaboration in the experiments described in chapter 6.

I would particularly like to thank Dr. D.B. Hope of this Department for allowing me to use his gel-slicing apparatus and Vitatron densitometer. Many others have helped in numerous ways, including Dr. L.O. Uttenthal, Dr. I. Benedeczky, Mr. I.W. Chubb and various members of the staff of the computing laboratory.

Mrs. B. Hicks aided me much with her skilful typing of this thesis and Mrs. Pamela Booth kindly drew some of the diagrams.

Finally, I thank the Medical Research Council for the award of a Training Scholarship.

CONTENTS

		Page
CHAPTER 1.	General introduction	1
CHAPTER 2.	Electrophoretic experiments on chromaffin granule proteins..	21
CHAPTER 3.	Gel-filtration of SDS-dissolved chromaffin granule membrane proteins	56
CHAPTER 4.	Discussion of the electrophoretic and gel-filtration experiments	79
CHAPTER 5.	Electrophoretic Studies of the Proteins of Noradrenergic Vesicles from Bovine Splenic Nerve Axons.	99
CHAPTER 6.	Studies of protein synthesis in sympathetic ganglia.	113
APPENDIX	. . . . .	184
REFERENCES		

INDEX TO FIGURES.

		Facing page
2.1.	Molecular weight calibration graph for 5% gels . .	38
2.2.	Ferguson plot of chromaffin granule membrane proteins	41
2.3.	Molecular weight calibration graphs for reduced and unreduced proteins on 6% gels	43
2.4.	Diagramatic comparison of staining patterns of chromaffin granule membrane proteins under different conditions	45
2.5.	Diagramatic comparison of the mobilities of soluble and membrane proteins in 6% gels	47
2.6.	Densitometric scans of amido black-stained chromaffin granule membrane proteins in 6% gels. .	49
2.7.	Densitometric scans of gels stained for protein and carbohydrate content	50
3.1.	Gel-filtration of SDS-dissolved chromaffin granule membranes on columns packed with Sephadex G-200 .	61
3.2.	Gel-filtration on small columns of Agarose gels. .	63
3.3.	The effect of lipid-extraction on gel-filtration on Sepharose 6B.	65
3.4.	Preparative gel-filtration on the large Sepharose 6B column.	66
3.5.	The fluorescence emission spectrum of the unknown material from the last peak of the gel-filtration experiment, measured at various excitation wavelengths.	70
3.6.	Second preparative gel-filtration on the large column	71
3.7.	Rechromatography of pooled "Fraction A".	72

Facing page

3.8. Rechromatography of pooled "Fraction B". 74

3.9. Rechromatography of pooled "Fraction C". 75

3.10. Molecular weight calibration graph for gel-filtration on Sepharose 6B 77

6.1. Labelling-patterns obtained after different soluene-extraction times. 138

6.2. The labelling-pattern from an experiment showing an anomalously low recovery of radioactivity . . . 139

6.3. The labelling-pattern from resting ganglia incubated for 2.5 hours with ^{14}C-leucine 143

6.4. The labelling-pattern at 0.6mm resolution 146

6.5. The labelling-pattern at 1.0mm resolution 147

6.6. Representation of the labelling-pattern as a continuous curve 148

6.7. Model labelling-profile calculated by the program GAUSSCALC. 154

6.8. Examples of output from the program CHOP 156

6.9. The labelling-pattern from normal, resting ganglia incubated for 2.5 hours with ^{3}H-leucine 157

6.10. An earlier labelling-pattern obtained from normal resting ganglia by incubation with ^{3}H-leucine. . . 158

6.11. The labelling-pattern from normal, resting ganglia incubated for 20 minutes with ^{3}H-leucine 159

6.12. ^{14}C-leucine labelling-patterns from resting and electrically-stimulated normal ganglia 161

6.13. ^{14}C-leucine labelling-patterns from resting and carbachol-stimulated denervated ganglia. 162

INDEX TO TABLES.

Facing page

2.1. Relative mobilities and estimated molecular weights of chromaffin granule membrane proteins after reduction and electrophoresis on 5% polyacrylamide gels (molecular weights calculated from the calibration graph in figure 2.1). 39

2.2. Relative mobilities and estimated molecular weights of unreduced chromaffin granule membrane proteins submitted to electrophoresis in 6% gels (calibration graph: fig. 2.3). 44

2.3. Mean molecular weights and staining intensities of chromaffin granule membrane proteins 46

2.4. Molecular weight estimates for unreduced chromaffin granule membrane proteins from 6% gels. 52

3.1. K_{av} and molecular weights of standard and SDS-dissolved chromaffin granule membrane proteins from chromatography on Sepharose 6B. 78

5.1. Relative mobilities and staining intensities of soluble proteins from bovine splenic nerve fractions FII and FIII. 106

5.2. Relative mobilities and staining intensities of SDS-solubilised membrane proteins from bovine splenic nerve fraction FII and FIII in 6% polyacrylamide gels (phosphate/SDS buffer) . . . 107

5.3. Relative mobilities and staining intensities of SDS-solubilised membrane proteins from re-purified bovine splenic nerve fractions RII and RIII and chromaffin granules (6% gels) 108

6.1. The percentage of radioactivity in ganglion samples which was not sedimented by TCA-treatment and centrifugation 125

6.2. Relative mobilities and staining intensities of ganglion proteins in 6% polyacrylamide gels . . . 126

6.3. Estimation of sectioning error in gel-slicing . . 128

6.4. Calculated "dpm per slice" for model gel, containing two peaks of radioactive protein. 150

6.5. Electrophoretic properties of rat adrenal chromaffin granule and ganglion proteins 163

6.6. Free amino acid concentrations in rat ganglia (from Table 1 of McBride & Klingman, 1972) 177

INDEX TO PLATES.

Facing page

2.1. Electrophoresis of unreduced chromaffin granule membrane proteins in gels of various total acrylamide contents. 42

2.2. Electrophoretic patterns of soluble and insoluble chromaffin granule proteins on 6% gels 48

2.3. The electrophoretic pattern of dopamine β-hydroxylase before and after reduction with dithiothreitol 55

3.1. Electrophoretic patterns of selected fractions of the effluent from the preparative gel-filtration 67

3.2. Electrophoretic analysis of the material eluted in selected tubes from the rechromatography of "Fraction A" 73

5.1. Electrophoretic patterns of the soluble proteins from bovine splenic nerve fractions 102

5.2. Electrophoretic patterns of soluble proteins from repurified nerve vesicle fractions. 104

5.3. Comparison of electrophoretic patterns of fractions FII and FIII with bovine serum proteins 105

5.4. Electrophoretic patterns of the insoluble proteins from bovine splenic nerve fractions. 107

5.5. Electrophoretic patterns of the insoluble proteins from repurified nerve vesicle fractions. 108

6.1. The stained band-pattern of SDS-solubilised ganglion proteins 127

CHAPTER 1.

General Introduction

	Page
Early studies of catecholamines in the adrenal medulla . .	1
Biosynthesis and storage of medullary catecholamines . . .	2
The composition of isolated chromaffin granules.	7
(a) Non-protein components.	7
(b) Protein components.	8
Chromaffin granule function.	13
Storage particles in sympathetic nerves.	15
Isolation of noradrenergic vesicles from axons	17
The cell bodies of sympathetic nerves.	19

This thesis is concerned with studies of the protein components of the amine-storing particles found in the chromaffin cells of the adrenal medulla (where they are called chromaffin granules) and of the closely-related vesicles present in the cell bodies and axons of sympathetic nerves. It has been established embryologically and histologically that adrenal medullary chromaffin cells and sympathetic nerve cells are derived from common parent cells, termed "primitive sympathetic cells", which probably originate in the neural crest (for a review, see: Coupland, 1965), and the biochemical, physiological and pharmacological similarities between these two tissues are a reflection of their common ancestry. Historically and for experimental reasons, many of the investigations of sympathetic nerves have been preceeded by studies of adrenal medullary chromaffin cells, and much of our understanding of the nature and functioning of sympathetic nerves is drawn from the analogy between these two systems.

Early Studies of Catecholamines in the Adrenal Medulla.

Coupland (1965) has defined a chromaffin cell as: "an element derived from neuro-ectoderm, innervated by pre-ganglionic sympathetic nerve fibres, capable of synthesising and secreting catecholamines and storing them in sufficient quantities to give a positive chromaffin reaction with aqueous solutions of potassium dichromate or chromate" and interest in the chromaffin tissue of the adrenal gland originally centred on its histochemical properties, following the early reports

of specific staining by ferric chloride (Vulpian, 1856) and potassium dichromate (Henle, 1865). The brown colouration seen after incubation with dichromate solution later gave rise to the term "chromaffin" (i.e.: chromium-binding; Kohn, 1900), used to denote cells showing this specific staining reaction.

The potent cardiovascular actions of extracts of adrenal medulla were discovered by Oliver & Schäfer (1895) and within a few years one of the active compounds causing this effect had been obtained in crystalline form and named adrenaline (Takamine, 1901). Both adrenaline and noradrenaline were synthesised shortly thereafter (Stolz, 1904; Dakin, 1905) and Elliot suggested that adrenaline might act as a chemical transmitter of nerve impulses as early as 1904; however, the identification of the neurotransmitter substance in sympathetic nerves was not achieved until 1946, when von Euler showed it to consist mainly, or exclusively, of noradrenaline.

Biosynthesis and Storage of Medullary Catecholamines.

It has taken many years to establish experimentally the route whereby adrenaline is synthesised in adrenal medullary chromaffin cells, but the sequence of chemical reactions now accepted as correct was suggested by Blaschko (1939) one year after the discovery by Holtz, Heise & Lüdtke (1938) of the first known enzyme in the pathway: dopa decarboxylase (also called "aromatic amino acid decarboxylase" since it acts on rather a wide variety of substrates), initially isolated from mammalian kidney but later

shown (Langemann, 1951) to be present in quantity in bovine adrenal medulla. The amino acid dopa is not itself the starting-point in the biosynthetic pathway and is formed *in vivo* from dietary tyrosine, by the action of the enzyme tyrosine hydroxylase (Nagatsu, Levitt & Udenfriend, 1964), this transformation being thought to be the rate-limiting step in the pathway under normal circumstances (Udenfriend, 1966). The dopamine thus formed from this dopa is converted to noradrenaline by the action of the enzyme dopamine β-hydroxylase, a copper-containing mixed-function oxidase isolated by Friedman & Kaufman (1965), which, like dopa decarboxylase and tyrosine hydroxylase, is present in sympathetic nerves as well as in adrenal medulla. The final conversion of noradrenaline to adrenaline is catalysed by the enzyme phenylethanolamine N-methyl transferase and occurs in the adrenal medulla, sympathetic nerves lacking this enzyme.

The independent discovery in 1953 by Blaschko & Welch and Hillarp, Lagerstedt & Nilson that 70 - 80% of the catecholamines in homogenates of bovine adrenal medulla could be sedimented by ultracentrifugation showed that these amines were present in subcellular particles. Although these particles appeared to have similar properties to mitochondria on differential centrifugation, they were soon shown to sediment to higher densities on centrifugation through a sucrose density-gradient (Blaschko, Hagen & Hagen, 1957) and their separate identity was confirmed by electron-microscopic

analysis of the density-gradient fractions (Hagen & Barrnett, 1960). The evidence for the existence of these separate catecholamine-containing granules (chromaffin granules) has been reviewed in detail (Smith, 1968 and Smith & Winkler, 1969) and will not be discussed further.

Reliable chemical studies of these amine-containing chromaffin granules are only possible if the granules can be isolated in a pure state, free of contamination by other cell particles, and several methods have been described for their preparation, although not all of these published methods actually provide the homogeneous preparation that is desirable. Methods used to prepare chromaffin granules include centrifugation through hypertonic sucrose density-gradients (Smith & Winkler, 1966), discontinuous density-gradients (Smith & Winkler, 1967a; Helle, Flatmark, Serck-Hanssen & Lönning, 1971), and isotonic sucrose-ficoll-D_2O density-gradients (Trifaró & Dworkind, 1970), as well as by differential centrifugation in isotonic sucrose (Banks, 1966; Taugner & Hasselbach, 1966) and by filtration through membrane-filters (Oka, Ohuchi, Yoshida & Imaizumi, 1966; Poisner & Trifaró, 1967). The most highly purified preparation, judged from the amounts and distributions of "marker" enzymes (characteristic of structures other than chromaffin granules), would appear to be afforded by the discontinuous density-gradient method of Smith & Winkler (1967a), which yields a pellet containing 48% of the catecholamines present in the low-

speed supernatant but only 7% of the lysosomes, 3% of the microsomes and less than 2% of the mitochondria.

A granule fraction isolated by a similar method by Helle et al., (1971) has been claimed to be less contaminated than the pellet yielded by the method of Smith & Winkler (1967a), but this claim is based on only fragmentary assays of marker enzymes and on estimates made on morphological grounds, and since the granule fraction in this study was found to contain appreciable glucose 6-phosphatase activity, normally associated with the presence of microsomal elements, some doubt remains as to the real extent of the "improvement" in purity of the preparation. The isotonic sucrose-ficol-D_2O density-gradient method of Trifaró & Dworkind (1970) certainly gives a less highly-purified chromaffin granule fraction, but in some cases the disadvantage of the presence of small amounts of contaminating material may be outweighed by the advantage that the chromaffin granules have not been exposed to hypertonic solutions during their isolation (which makes them osmotically fragile). This advantage is shared by granules prepared by the membrane-filtration methods, but it has been shown that the Millipore-filtration procedure of Oka et al., (1966) gives a granule fraction which is heavily contaminated by mitochondria (Trifaró & Dworkind, 1970), and this is probably true of the method of Poisner & Trifaró (1967) as well, indicating that these preparations are not suitable for detailed studies.

The chromaffin granules prepared from homogenates of adrenal

medullary tissue by these means are not homogeneous in respect of their catecholamine content. Morphologically, both noradrenaline-containing and adrenaline-containing chromaffin cells have been distinguished in the adrenal medullae of various species and the proportion of each is species-dependent. In the electron-microscope, adrenaline-containing chromaffin cells are distinguished by their content of moderately electron-dense chromaffin granules, seen after fixation with glutaraldehyde and osmium tetroxide, whereas the chromaffin granules of noradrenaline-containing chromaffin cells are highly electron-dense after this treatment (see, inter alia: Grynszpan-Winograd, 1969 and Benedeczky & Smith, 1972; also Coupland, 1965, which contains much information on the light and electron-microscopic features of chromaffin cells).

The morphological heterogeneity of the chromaffin cells is paralleled by the biochemical finding of various ratios of adrenaline to noradrenaline in the adrenal medullae of different species (for details of the distribution of catecholamines in almost every conceivable vertebrate, see: Holzbauer & Sharman, 1972). In bovine adrenal medulla, 74% of the catecholamine is present as adrenaline (West, 1955), while the ratio falls to 51% in pig adrenal and is as high as 98% in rabbit adrenal.

Noradrenaline-containing chromaffin granules are more dense than those containing adrenaline and they sediment to a higher sucrose concentration on density-gradient centrifugation (Eade,

1957). This fact enabled Winkler (1969) to prepare a highly-purified fraction of noradrenaline-containing chromaffin granules from pig adrenal medulla. Although the lysolecithin content of these noradrenaline-containing granules was higher than that of "mixed" granules from either pig or ox adrenal medulla, the noradrenaline-containing granules were in other respects similar to the mixed granules. All the experiments described in this thesis were performed with preparations of "mixed" granules, which contain both adrenaline- and noradrenaline-granules.

The Composition of Isolated Chromaffin Granules.

(a). Non-protein Components.

Catecholamines make up 20.5% of the dry weight of bovine adrenal chromaffin granules, but more remarkable than this is the fact that adenine nucleotides, mainly adenosine triphosphate (ATP), make up another 15% of the dry weight (both figures are from Hillarp, 1959). This ATP content is higher than that found in any other tissue component (Blaschko, Born, D'Iorio & Rade, 1956) and is therefore one of the distinctive features of the chromaffin granule. As will be described later, it has been suggested that this high ATP content is related to the storage-function of the granule.

Chromaffin granules contain only traces of fatty acids, triglycerides and cholesterol esters, but contain relatively large amounts of cholesterol and phospholipids and show a high molar ratio of cholesterol to lipid phosphorus of 0.58 (Blaschko, Firemark,

Smith & Winkler, 1967). The phospholipid fraction is composed of 26.0% lecithin, 36.1% phosphatidylethanolamine, 9.2% of (phosphatidylserine and -inositol), 10.9% of sphingomyelin, 0.6% of phosphatidic acid and/or cardiolipin and, most unusually, 16.8% of lysolecithin. The very high content of lysolecithin is another characteristic feature of chromaffin granules and may have a role to play in exocytosis when the membrane of the granule fuses with the plasma membrane (see later), since lysolecithin has a "fluidising" effect on membranes.

(b). Protein Components.

The major component of chromaffin granules, making up 35.0% of the dry weight, is protein, and this protein may be divided into two categories: soluble protein, which is released into solution, along with the catecholamine and ATP, on exposure of the isolated granules to dilute buffer, and membrane protein, which remains sedimentable and lipid-associated even after prolonged washing of the residue remaining after the release of soluble constituents by the hypo-osmotic shock with dilute buffer. Hillarp (1958c) found that 77% of the protein present in chromaffin granules isolated by sucrose density-gradient centrifugation was solubilised by hypo-osmotic shock and later workers have reported similar values (Winkler, Hörtnagl, Hörtnagl & Smith, 1970). Apart from dopamine β-hydroxylase activity, which is almost equally distributed between the soluble and insoluble protein fractions, no enzymic properties

have been established for the soluble protein and it has been speculated that this protein might be concerned with maintaining the high concentration of amines and ATP within the granules, by formation of some kind of ternary complex (Hillarp, 1958b).

The first attempt at isolating and characterising the soluble protein of chromaffin granules was described by Hillarp (1958a), who precipitated the protein by treatment with ethanol at pH 4. While he reported that this protein appeared to be electrophoretically homogeneous and had an isoelectric point of ca. pH 4, Hillarp did not actually present evidence supporting these claims. A partial resolution of this ethanol-precipitable protein was achieved by Blaschko & Helle (1963) and this work was described in greater detail by Helle (1966a). In this paper Helle described how the ethanol-precipitated soluble protein could be completely freed of nucleotide by passage through a column of Sephadex G-75 and reported the amino acid analysis of the (total) protein, showing it to contain an unusual preponderance of glutamate (25.4% by weight) and proline (8.2%). The protein was also shown to be heterogeneous on starch gel electrophoresis, but attempts to isolate individual pure proteins from this mixture were not successful.

Similar studies were undertaken at about the same time by Kirshner and co-workers, who dispensed with ethanol-precipitation and instead fractionated the proteins on DEAE-cellulose and

Sephadex G-200 columns, obtaining two major proteins after the latter step and finding them to have molecular weights of 21,000 and 39,000. (Smith, Kirshner & Kirshner, 1964; Kirshner, Holloway, Smith & Kirshner, 1966). In the same year, Blaschko, Smith & Winkler (1966) reported that the major component of the soluble protein of chromaffin granules had a molecular weight of 75,000 when separated by gel-filtration on Sephadex G-200 at low ionic strength and this preliminary report was followed by a fuller account of the isolation of this principal component (Smith & Winkler, 1967b) which was later termed "chromogranin A" (Schneider, Smith & Winkler, 1967). On the basis of results from high-speed membrane osmometry, Smith & Kirshner (1967) later revised their molecular weight value to 80,000, in agreement with the value of 77,000 quoted by Smith & Winkler (1967b). While the properties of chromogranin A have been described in some detail, particularly its unusual behaviour in gel-filtration and during ultracentrifugation in response to changes in its ionic environment (Smith & Winkler, 1967b), the other chromogranins have not been studied in detail, with the exception of the soluble form of the enzyme dopamine β-hydroxylase.

The presence of soluble dopamine β-hydroxylase in osmotic lysates of chromaffin granules was not recognised at first, since the adrenal medulla contains endogenous inhibitor(s) of this enzyme (Duch, Viveros & Kirshner, 1968). It was however recognised that

this enzyme was present in the membrane (insoluble protein) fraction and recent studies (Hörtnagl, Winkler & Lochs, 1972) have shown that there are no detectable differences between the soluble and membrane-bound forms of this enzyme.

There have been several reports that chromogranin A is a lipoprotein (Helle, 1968; Helle & Serck-Hanssen, 1969; Mylroie & Koenig, 1971). None of these claims can be considered to hold up under closer scrutiny. In order to demonstrate the "lipoprotein" nature of the chromogranins, it has been found necessary to lyse chromaffin granules either by sonication (which may produce small fragments of membrane not sedimented on centrifugation for short periods) or by treatment with the detergent Triton X-100, which is known to solubilise both proteins and lipids from the membrane fraction.

Comparative immunochemical and electrophoretic studies of the soluble proteins from chromaffin granules from ox, horse, pig, sheep and human adrenal have demonstrated similarities or identity between the chromogranins from each of these species (Winkler, Zeigler & Strieder, 1966; Helle, 1966b; Strieder, Ziegler, Winkler & Smith, 1968). In addition, the proteins of chromaffin granules from a human phaeochromocytoma (a tumour of chromaffin cells) were shown to be identical with those from normal human adrenal (Strieder et al., 1968), and the chromogranins of the noradrenaline-containing chromaffin granules from pig adrenal medulla were shown to be the same as those from mixed granules on electrophoresis and by amino

acid analysis (Winkler, 1969).

The insoluble (membrane) proteins of chromaffin granules have been studied rather less fully than the soluble proteins, and one of the objects of the work described in this thesis was to further characterise the major membrane proteins (termed "chromomembrins" by Winkler, 1971). The presence of an ATPase in the granule membrane was noted as early as 1958 (Hillarp, 1958a) and the membrane-bound enzyme was further investigated by Banks (1965), who found that it was activated by Mg^{++}, but this component has proved to be one of the most difficult to study and only recently has any progress been made towards obtaining the enzyme in solution in active and homogeneous form. (Trifaró & Warner, 1972). The other major membrane protein which has so far resisted purification is the cytochrome b_{559}, described by Banks (1965) and Ichikawa & Yamano (1965) and also found in the microsomal fraction from homogenates of bovine adrenal medulla (Banks, 1965; see also an earlier abstract by Spiro & Ball, 1958). This protein has been suggested to form part of an electron-transport chain in the granule membrane (Flatmark, Terland & Helle, 1971) but has not been purified. As will be described in chapter 3 of this thesis, it is probable that the heme group which gives this protein its characteristic visible absorption spectrum is relatively easily dissociated from the protein moiety during attempts at isolation of the pure protein.

The two major proteins of chromaffin granule membranes were

identified electrophoretically by Winkler et al., (1970) and named chromomembrin A and chromomembrin B. The first of these (chromomembrin A) has been shown to be the membrane-bound form of the enzyme dopamine β-hydroxylase (Hörtnagl, Winkler & Lochs, 1972), but no enzymic activity has yet been found for chromomembrin B. Both of these proteins were purified by gel-filtration on Sephadex G-200 (Hörtnagl, Winkler, Schöpf & Hohenwallner, 1972) and it was their work which stimulated the present investigations.

Chromaffin Granule Function.

The functions of chromaffin granules in adrenal medullary chromaffin cells would appear to be twofold: firstly, they act as the site of storage of the catecholamine hormones and, secondly, they participate in the process by which these amines are released into the extracellular environment in response to stimulation. Both of these functions have been the objects of numerous studies and while the mechanism(s) by which the large quantities of amines are retained in the granules are not completely clear, it has been established that the release of at least the major part of the catecholamines occurs by the process termed "exocytosis", in which the membrane of the chromaffin granule fuses with the plasma membrane of the chromaffin cell and the contents of the granule are expelled through an opening in the membrane at the point of contact. Both of these aspects of the function of chromaffin granules have been very extensively reviewed (Smith, 1968; Kirshner & Kirshner, 1971;

Smith & Winkler, 1972 and Smith, 1972a) and only the main conclusions will be summarised here.

Both active and passive processes have been proposed to be involved in the storage of the catecholamines and it is probable that both contribute to the maintenance of the amine stores *in vivo*. The observation that the molar ratio of catecholamine to ATP in isolated granules was 4 - 5 : 1 led to the suggestion (Blaschko et al., 1956) that the amine might be stored inside the granules as an electrically neutral complex, in which four molecules of the catecholamine were complexed with each molecule of ATP. Such a complex has been shown to form at low temperatures (ca. 0°) in the presence of divalent cations (Berneis, Da Prada & Pletscher, 1970), although it dissociates as the temperature is raised to 37°. By contrast, the membranes of the chromaffin granules have been shown to contain an active-uptake mechanism which transports catecholamine with accompanying hydrolysis of ATP (Taugner, 1971; Taugner, 1972) and recent experiments by Baumgartner, Winkler & Hörtnagl (1973) suggest that both complex formation and active uptake are probably important under physiological conditions.

Although "exocytotic profiles" showing fusion of granule and plasma membranes are only occasionally seen in electron-micrographs of adrenal tissue, except in sections from the adrenal medulla of the golden hamster (see e.g.: Benedeczky & Smith, 1972), the hypothesis that exocytosis was the release-mechanism in the medulla

was first proposed by electron-microscopists (De Robertis & Vaz Ferreira, 1957) and has been established beyond reasonable doubt by biochemical studies of isolated perfused organs. Thus, on stimulation of perfused medulla, catecholamines, adenine nucleotides and chromogranins are recovered in the effluent from the gland in ratios similar to those found in isolated chromaffin granules, whereas components of the cell cytoplasm are not released. The exact mechanism by which the chromaffin granule membrane and that of the cell surface fuse has not been elucidated (and is unlikely to be fully understood until purified preparations of the plasma membrane are available for analysis) and the suggestion of a possible involvement of lysolecithin in this process is, as yet, speculation. A particularly full discussion of each of the distinct steps in the exocytotic process is to be found in Smith & Winkler, 1972.

Storage Particles in Sympathetic Nerves.

Like adrenal medullary chromaffin cells, sympathetic nerve cells synthesise, store and secrete catecholamines. As in the adrenal medulla, the catecholamines are stored within membrane-limited particles. Once again, very full reviews are available which are concerned with the storage (Geffen & Livett, 1971; Smith, 1972b) and release (Smith & Winkler, 1972; Smith, 1972c; De Potter, Chubb & De Schaepdryver, 1972; Smith, 1973) of the catecholamines and I shall not attempt a detailed discussion of the evidence for

the existence of specific adrenergic vesicles within nerves or for release of the neurotransmitter by the exocytotic mechanism.

Although there are strong biochemical similarities between sympathetic nerves and the adrenal medulla, the nerve cell has certain specialised roles to play and its shape and organisation differ considerably from the adrenal medullary "model" of a typical catecholamine-containing cell. Morphologically and functionally, sympathetic neurons may be considered to be composed of three different but continuous parts: the cell body (or "perikaryon"), the nerve trunk (or "axon") leading out from the cell body, and the terminal region. The distinguishing feature of the sympathetic neuron is the fact that the site of synthesis of the catecholamine storage-particles in the cell body is extremely remote from the site of release of the catecholamines at the terminal, from which it may be separated by a distance of as much as 10^5 cell body diameters. Secondly, although the proteins of the noradrenergic nerve-vesicles are synthesised in the cell body (Droz, 1969), only a trace amount of the noradrenaline present in the nerve terminals is supplied from the perikaryon, the majority being synthesised locally (Geffen & Rush, 1968).

The non-uniformity of the nerve cell is reflected in other important facts. Although the cell body and axon contain only one kind of noradrenaline-containing particle, the so-called "large dense-cored vesicle" which has a diameter of ca. 70 nm and is thought

to be the "heavy" noradrenergic vesicle observed on sucrose density-gradient centrifugation of nerve homogenates, nerve terminals contain both large and small dense-cored vesicles, the latter, with diameters of ca. 44 nm, containing the bulk of the noradrenaline and being the predominant type of vesicle - probably identical with the "light" noradrenergic vesicle seen in density-gradients. Furthermore, the heavy (large) vesicles in the nerve terminals have been shown (De Potter, 1971) to contain 10 times more noradrenaline, when expressed relative to their dopamine β-hydroxylase activity, than the corresponding "heavy" vesicles in the non-terminal axons.

Isolation of Noradrenergic Vesicles from Axons.

The development of methods for the preparation of bovine adrenal medullary chromaffin granules in a highly pure state yielded considerable dividends in terms of the knowledge gained about the structure and composition of these granules and it is hardly surprising that repeated efforts have been made to obtain preparations of the noradrenergic vesicles of sympathetic nerves of similar purity. Unfortunately, these attempts have so far proved only partially successful and there is little doubt that even the best preparations are still considerably contaminated with other cellular particles and fragments. Apart from some experiments on density-gradient centrifugation of vesicles thought to be derived from nerve terminals and a recent study of the particles of bovine stellate ganglion cell body (Chubb, De Potter & De Schaepdryver, 1972), most experiments have been carried out on

homogenates of bovine splenic nerve trunks (axons).

Careful subcellular fractionation studies of splenic nerve homogenates were made by Hörtnagl, Hörtnagl & Winkler (1969) and and by De Potter, Smith & De Schaepdryver (1970) and Lagercrantz, Klein and Stjärne (1970) published a purification method for the noradrenergic vesicles which gave a preparation in which they believed, from biochemical evidence, that 10% of the total protein was present in vesicles. Lagercrantz later claimed (Lagercrantz, 1971) that this vesicle preparation was, in fact, about 40% pure, but this claim was based on examination of the sample by electron microscopy and this technique may not give a very reliable estimate of the degree of contamination.

Although this granule fraction is clearly far from pure, Flatmark, Lagercrantz, Terland, Helle & Stjärne (1971) claimed, from direct analysis, to have shown the presence of the same flavoproteins, cytochrome b_{561} and NADH: (acceptor) oxidoreductase activity in nerve vesicles as were earlier found in chromaffin granule membranes. From an examination of their distributions on density-gradient and differential centrifugation, Hörtnagl et al., (1969) and De Potter et al., (1970) were able to show ATP, dopamine β-hydroxylase and chromogranin A to be present in the noradrenergic nerve-trunk vesicles. Banks, Helle & Mayor (1969) had earlier been able to demonstrate the presence of a chromogranin-like protein in the pressed-out sap from splenic nerves by an immunochemical

method, but they had not been able to show conclusively that this protein was located in noradrenergic granules. An immunological method was also used by Hörtnagl, Winkler & Lochs (1973) to show that chromomembrin B was located in the nerve granules.

The Cell Bodies of Sympathetic Nerves.

It has already been mentioned that whereas the perikaryon of the sympathetic nerve does not contribute a significant amount of noradrenaline to the store in the terminal, since the axoplasmically transported nerve granules are relatively deficient in noradrenaline, it does supply the soluble proteins which are secreted from the terminals and also the membrane proteins of the vesicles which transport this soluble protein. Although it has been clear for a long time that the generation of action potentials in sympathetic nerves, which takes place at the cell body, is under the control of presynaptic elements (both excitatory and inhibitory), it has only recently been shown that, in the longer term, the levels of various enzymes in the cell body are also governed by the activity of the incoming nerve fibres. The variations in enzyme levels have been shown to be due to changes in the rates of synthesis, rather than intrinsic activities, of the enzymes and the phenomenon has been named "trans-synaptic induction". (see: Thoenen, 1972 for a recent review of this topic). It was the demonstration that the rate of protein synthesis in ganglion cell bodies may be modulated by the activity of the pre-ganglionic nerves which encouraged Professor

Toschi and I to make the experimental studies described in chapter 6 of this thesis.

To summarise briefly, the studies on which this thesis is based are aimed at gaining further information about the nature of the membrane proteins of bovine adrenal chromaffin granules (chapters 2, 3, and 4), to characterise further the related proteins present in noradrenergic vesicles from bovine splenic nerves (chapter 5) and to investigate what factors may influence the synthesis of these proteins in the cell bodies of sympathetic nerves (chapter 6). For convenience, the description of the electrophoretic experiments carried out on chromaffin granule membrane proteins has been separated into chapter 2, away from the account of the attempts to purify these proteins by gel-filtration which form chapter 3, but the results of both of these series of experiments are discussed together in chapter 4, since they both bear on the same fundamental topic.

CHAPTER 2.

Electrophoretic Experiments on Chromaffin Granule Proteins.

Page

Methods

Preparation of chromaffin granules. 21

Preparation of the chromaffin granule membranes 22

Solubilisation of the granule membrane. 24

Polyacrylamide gel electrophoresis of SDS-dissolved membrane proteins. 25

Quantitative densitometry of stained polyacrylamide gels. . 33

Materials 35

Results 36

Molecular weight studies 37

Reduced proteins. 38

Unreduced proteins. 42

Soluble chromaffin granule proteins 45

Quantitative densitometry of chromogranin A 47

Membrane glycoproteins 49

An alternative approach for calculating molecular weights . 51

Reduction of dopamine β-hydroxylase/chromomembrin A 53

METHODS

Preparation of Chromaffin Granules.

The method used, throughout the experiments described in this thesis, for the preparation of bovine adrenal chromaffin granules was identical with that described by Winkler, Hörtnagl, Hörtnagl and Smith (1970) which is based on the method described by Smith and Winkler (1967a). Using this procedure, cortex-free bovine adrenal medullae were chopped finely with a knife and homogenised in a Potter-Elvehjem glass homogeniser (Kontes Glass Company, Vineland, New Jersey, U.S.A.) with ice-cold 0.3M sucrose solution (volume-to-weight ratio of ca. 5:1), using a Teflon pestle (clearance 0.08mm) rotated at 950 r.p.m. by an electric motor. Homogenisation was continued until the pestle had been passed fully up and down a total of five times. The homogenate was centrifuged at 2°C at low speed (380 g_{av}) for 20 minutes, giving a sediment, which was discarded, and a supernatant (referred to as "low speed supernatant").

25ml. aliquots of this supernatant were centrifuged at 2°C in the A30 rotor of the ultracentrifuge (either a Spinco L50 or a Beckman L2-65B) at 10,000 r.p.m. (8,720 g_{av}) for 20 minutes. This yielded a "medium speed supernatant" and a pellet which was resuspended with 20ml of 0.3M sucrose and re-centrifuged under the same conditions.

The pellet from this re-centrifugation was used as starting

material for further purification of chromaffin granules. For this purpose, each pellet was re-suspended with 2.0ml of cold 0.3M sucrose, taking care not to disturb the small "button" of red blood cells. Aliquots of up to 3.0ml of this re-suspended large granule fraction were layered onto 7.5ml portions of ice-cold 1.6M sucrose solution in polyallomer tubes and the tubes were then centrifuged at 2°C in the 40 rotor of the ultracentrifuge at 35,000 r.p.m. ($80,790g_{av}$) for 60 minutes.

After this centrifugation, the chromaffin granules formed a tightly-packed pellet at the bottom of each tube. The supernatant and a loose sediment (the "fluffy layer") overlaying the pellet could then be poured off without disturbing this pellet, which was washed twice by swirling with 1.0ml quantities of 1.6M sucrose. This pellet is only slightly contaminated by mitochrondria, lysosomes and other organelles, as judged by enzymatic analyses (F. Dubois, B.Sc. Thesis, Oxford, 1970), and will be referred to as "chromaffin granules".

Preparation of the Chromaffin Granule Membranes.

Exposure of chromaffin granules prepared by the above method to hypo-osmotic shock, accompanied by slow freezing and thawing, can be used to disrupt the particles, producing soluble and insoluble fractions. These fractions are assumed to correspond to the contents and to the membranes of the chromaffin granules, respectively.

Catecholamines are rapidly oxidised in aqueous solution above pH7 and so a buffer of pH5.9 is usually employed to lyse the

chromaffin granules. A suitable buffer is that described by Smith and Winkler (1967b), made by dissolving 6.057g of tris and 5.905g of succinic acid in water, titrating the solution to pH5.9 with sodium hydroxide solution and making up to 1 litre. A ten-fold dilution of this stock solution gives a solution which is 0.005M in tris and succinate and it was this diluted buffer which was used to lyse the chromaffin granules.

Each pellet of highly-purified chromaffin granules was suspended with 5ml of (diluted) buffer, using a pipette. This suspension was frozen in a deep-freeze at -20°C and then thawed by standing the centrifuge tube in water at room temperature. After the suspension had thawed, it was centrifuged at 30,000 r.p.m. ($59,360g_{av}$) in the 40 rotor of the ultracentrifuge for 60 min. The supernatant (termed the "soluble lysate") was decanted and the sediment was resuspended, frozen at -20°C, thawed and centrifuged again, as above. This procedure was repeated a further three times, in order to remove the bulk of the soluble components of the chromaffin granules (Winkler et al., 1970).

The insoluble residue obtained in this way is termed "chromaffin granule membranes" and was found to consist of empty sacs and sheets of membrane, when examined in the electron microscope. Chemical analysis (Winkler et al., 1970) has shown this membrane residue to contain c.22% of the total protein and virtually all of the cholesterol and phospholipids of the original chromaffin granules.

Solubilisation of the Granule Membrane.

For electrophoresis and gel-filtration experiments the chromaffin granule membrane pellets, prepared as described above, were dissolved by treatment with the detergent sodium dodecyl sulphate (hereafter abbreviated to SDS). This detergent has been shown to give the most efficient solubilisation of both protein and lipid (Winkler et al., 1970) but has the disadvantage that it completely inactivates both the dopamine β-hydroxylase and the magnesium-stimulated adenosine triphosphatase in the granule membrane.

Membrane pellets, each containing 3 - 5mg of protein, were solubilised for electrophoresis by suspension with 1 - 2ml of a solution containing 1%(w/v) SDS and c.10%(w/v) sucrose in 0.01M sodium phosphate buffer pH6.5 (see later). (Note: throughout this thesis all percentage compositions are w/v unless noted otherwise). The tube containing the suspension was then immersed in a bath of boiling water for 5 minutes. After this, the suspension was allowed to stand at room temperature overnight before being centrifuged, at room temperature, in the 40 rotor of the ultracentrifuge at 30,000 r.p.m. ($59,360g_{av}$) for 60 minutes, giving a clear supernatant and a small pellet (which was discarded). If the solubilised proteins were to be separated by gel-filtration, the suspending medium was instead column buffer (see later) which had been made 1% in SDS and it contained no sucrose; otherwise the procedure was as just

described.

Polyacrylamide Gel Electrophoresis of SDS-dissolved Membrane Proteins.

Polyacrylamide gels were made by a procedure based on the simple method described by Clarke (1964). A commercial monomer mixture - Cyanogum 41, which is composed of 95%(w/w) acrylamide and 5%(w/w) N,N'- methylene bisacrylamide - was used to make the gels in all experiments. The following solutions, mixed in the volume ratios 1:2:1:4, were used to prepare the polymerisation mixture, 1.2ml aliquots of which were pipetted into glass "running tubes" (6mm i.d. x 60 mm), overlayered with distilled water and allowed to set:-

1). 0.28%(v/v) aqueous N.N.N',N'-tetramethylethylenediamine (TEMED)

2). An aqueous solution of Cyanogum 41 at a concentration four times greater than that desired in the polymerised gels.

3). A solution containing 0.8% SDS in 0.8M sodium phosphate buffer pH6.5. (SDS is not sufficiently soluble in phosphate buffer to give the required concentration at room temperature and so this solution must be warmed slightly before use.)

4). An 0.14% aqueous solution of ammonium persulphate. (This solution was de-aerated for a few minutes, by applying a vacuum generated by a water-pump, before it was added (last) to the gel mixture, to initiate the polymerisation reaction. This solution and the TEMED solution (1) were made up freshly at least every week.)

The Cyanogum (2) was not kept as a stock solution, but was

instead made up by weighing out the monomer powder on each occasion, as required.

Stock sodium phosphate buffer pH6.5 (1M in phosphate) was made by dissolving 81.0g of anhydrous disodium hydrogen orthophosphate and 67.0g of sodium dihydrogen orthophosphate (dihydrate) in distilled water, to give a final volume of 1 litre. (see: Maizel, 1969). Suitable dilutions of this stock buffer were used to make the phosphate/SDS solution for the polymerisation mixture (see above: 3) and also the electrode buffer for the electrophoresis.

Generally, polymerisation of the acrylamide monomer, indicated by the formation of a slightly opalescent gel with a sharp interface with the overlayering water, was evident 20 - 30 minutes after the persulphate solution (the initiator) had been added to the polymerisation mixture. The newly-formed gels were, however, always left overnight at room temperature before being used, to allow the reaction to go to completion. The following day, the excess water was shaken from the top of the gels and the gels, in their running tubes, were mounted in the electrophoresis apparatus and "pre-run", at a current of 5mA/gel, for at least 60 minutes, using an 0.1% solution of SDS in 0.1M sodium phosphate buffer pH6.5 as electrolyte. The pre-run serves to remove any persulphate ions remaining from the polymerisation step and was always performed before carrying out an electrophoretic separation on the gels. That such precautions may be necessary is indicated by the report of Schyns (1968) who reported artefact-

formation during electrophoretic separation of trypsinogen and suggested that this was due to the oxidation of disulphide bridges by persulphate ions.

Following the completion of the pre-run, the gel tubes were removed from the electrophoresis apparatus and the surface of the gel was dried with tissues. 2 - 5 microlitres of an aqueous solution of bromophenol blue (just sufficiently concentrated to give an easily visible band on completion of the electrophoretic separation) were applied to the top of each of the gels and the samples (volume: up to 100µl) were loaded on, using an Eppendorf automatic pipette (Eppendorf Gerätebau, Netheler & Hinz GmbH, 2000 Hamburg 63 (Hummelsbüttel), Barkhausenweg 1) and mixed with the dye by a rotatory shaking movement of the tube. After re-mounting the tubes in the upper chamber of the electrophoresis apparatus, electrolyte was very gently layered over the sample solutions. The electrode vessels were filled with fresh electrolyte (0.1% SDS in 0.1M sodium phosphate buffer pH6.5) and the separation was commenced, at an initial current of 5mA/gel. After 20 minutes, the dye band had penetrated into the upper part of the gel and the current was then increased to 8mA/gel. It was held constant at this value until the dye band had migrated to within a few millimetres of the end of the gel (after 2 - 2½ hours), when the run was stopped.

After demounting each tube from the upper electrode vessel, the length of the gel and the distance of migration of the dye band (from

the origin) were measured. The gel was then removed from its running-tube by "rimming" with a long needle, mounted on the end of a syringe full of water - turning the tube while gently expelling the water from the syringe. The gel was then "fixed" by immersing it, at room temperature, in 25% aqueous trichloroacetic acid in a tube on a reciprocating shaker. After 2 hours, the solution was discarded and the gel was stood overnight in a fresh quantity of fixative. The next morning, the gel was stained by shaking for 1 - 2 hours with a saturated (ca. 0.1%) solution of Amido Black dye (G.T. Gurr Ltd., Lane End Road, High Wycombe, Bucks) in 25% trichloroacetic acid (abbreviation: TCA). Excess dye was leached from the gel by shaking it in frequently-changed portions of 7%(v/v) acetic acid, until the protein bands were visible against an unstained back-ground. Routinely, the distance of migration of each band from the origin (which was the top of the gel) and the length of the gel were then measured, to the nearest 0.1mm, and visual estimates were made of the relative staining intensities of each of the bands. For quantitative purposes, the mobility of a particular protein band, relative to the mobility of the bromophenol blue marker dye band, is expressed in units of R_m x 100, defined as:

$$R_m \times 100 = \frac{\text{distance migrated by protein band}}{\text{distance migrated by dye band}} \times \frac{\text{length of gel before staining}}{\text{length of gel after staining}} \times 100.$$

In some experiments gels were treated with periodic acid and Schiff's reagent, to visualise carbohydrate-containing substances. After the initial fixation in TCA, the gels were shaken in a 1% solution of periodic acid in the TCA fixative for 1 hour and then washed by shaking with two successive portions of TCA fixative, for 30 minutes each. The gels were then left to stand overnight in fresh fixative and, the following morning, were given two further 30-minute washes before being immersed, in the dark, in commercial Schiff's reagent (BDH Chemicals Ltd.). Pink bands, indicating the presence of carbohydrate-containing material, developed within 30 - 60 minutes.

Standard proteins of known molecular weights were used to construct "calibration graphs", relating R_m x 100 to $\log_{10}$ (molecular weight). Each standard protein was dissolved separately in a solution containing 1.0% SDS and 10% sucrose in 0.01M sodium phosphate buffer pH6.5 (or the same solution containing, in addition, 1.0% dithiothreitol, if it was desired to reduce disulphide bonds) at a protein concentration of 1 - 2 mg/ml. The resulting solutions were left overnight at room temperature before use. Suitable quantities of these solutions could then be applied directly to the polyacrylamide gels; alternatively, if the mobilities of the proteins differed sufficiently from one another, several of these solutions could be mixed before submitting them to electrophoretic analysis.

Soluble proteins were examined by electrophoresis in 6% polyacrylamide gels with the discontinuous tris-glycine buffers

described by Clarke (1964). Details of the gel preparation were the same as above, except that the ammonium persulphate solution was not de-aerated before use and the sodium phosphate/SDS buffer was replaced by an equal volume of tris-glycine buffer, pH8.6. This tris-glycine ("gel") buffer was made by dissolving 6.0g of ARISTAR tris base and 29.0g of glycine in distilled water and making the volume up to 1 litre. This buffer was diluted 1 + 7 (v/v) to give electrolyte for the pre-run, which was carried out at a current of 1mA/gel, for at least 1 hour.

A tris-glycine-HCl buffer was used for the electrolyte during the separation of the proteins. It was made by dissolving 6.0g of ARISTAR tris base and 29.0g of glycine in water and adding 5.0ml of 1.0M hydrochloric acid before making the volume up to one litre. It had a pH of 8.1 and was diluted 1 + 9 (v/v) to give electrolyte for the separative run. The separation was begun at a current of 0.5mA/gel for 5 - 10 minutes, after which the current was increased to 1.0mA/gel and held constant until the conclusion of the experiment. Instead of being fixed in 25% TCA solution, gels were immersed in a solution containing 1.0% Amido Black dye in 7% (v/v) acetic acid, for 1 - 2 hours, and were then destained by leaching with acetic acid as described earlier.

Preparation of Soluble Dopamine β-hydroxylase and Chromogranin A.

Highly purified preparations of the soluble form of dopamine β-hydroxylase and of chromogranin A were needed for some experiments

and these were obtained from the "soluble lysate" mentioned earlier (see under "Preparation of the granule membranes"). The soluble lysate was transferred to 18/32" Visking dialysis tubing and was dialysed extensively at 2°C against the 0.005M tris-sodium succinate buffer (pH 5.9) used to lyse the granules. The non-diffusible contents of the dialysis bag were then concentrated to a volume of 3 - 5ml by ultrafiltration in an apparatus similar in design to that described by Sober et al. (1956). The concentrated protein solution was applied to a column of Sephadex G-200 (16cm^2 x 90cm) and eluted at 2°C with the same tris-sodium succinate buffer. Both the dialysis and elution buffer contained 0.02% sodium azide, added to inhibit the growth of bacteria. The elution profile of the soluble proteins on this column was similar to that described by Smith & Winkler (1967b), except that there was no non-protein peak at the void volume. The fractions making up the major peak, which contain chromogranin A and soluble dopamine β-hydroxylase, were pooled.

Ion-exchange chromatography on DEAE-Sephadex was used to separate the two component proteins of these pooled fractions. 2 - 3g of DEAE-Sephadex A-25 ion-exchange resin were swollen in distilled water and converted to the chloride ion form, by suspension with an excess of 0.1M hydrochloric acid. After washing with distilled water, the resin was equilibrated with starting buffer. The starting buffer was made by titrating a mixture of 10.0ml of 5.0M ARISTAR hydrochloric acid and ca. 900ml of distilled water to pH 7.5 by

gradually adding a solution of 1.0M tris base, 55 - 60ml being needed, and making up to 1 litre. The resin suspended in this buffer, was packed into a small glass column (diameter 1cm) to give a bed-height of 10cm of resin (corresponding to a volume of ca. 8ml) at a flow-rate of 25ml/h.

The pooled material from the G-200 column was pumped through the column, under which conditions it was firmly bound by the ion-exchanger. The column was then pumped with a further 60ml of starting buffer before applying a linear concentration gradient of sodium chloride (limit buffer: 0.8M sodium chloride, made up in starting buffer). Fractions of ca. 2ml were collected during the gradient elution stage. Dopamine β-hydroxylase was eluted as a small UV-absorbing peak at a salt concentration of 0.05M NaCl. (Salt concentrations were measured by atomic absorption spectrometry of 500 x diluted samples, using an Unicam SP 90 atomic absorption spectrometer). The protein eluted in this peak is homogeneous, as judged by electrophoresis in 6% polyacrylamide gels using either tris-glycine or sodium phosphate/SDS buffer systems.

The maximum of the second UV-absorbing peak occurred at a sodium chloride concentration of 0.25M, but a long "tail" extended from the maximum, towards higher salt concentrations. The material eluted at the peak maximum was chromogranin A, together with a very small amount of dopamine β-hydroxylase, which could be detected by electrophoresis if very high protein loadings were used.

Chromogranin A was the major protein throughout the rest of the peak, but later fractions contained small quantities of additional components, presumably other chromogranins, which appear as faster running components on electrophoresis with either buffer system and only the peak fractions were taken if chromogranin A of the highest purity was needed (as in the densitometric studies).

Quantitative Densitometry of Stained Polyacrylamide Gels.

Whenever measurements were made of the migration distances of individual, stained protein bands within polyacrylamide gels, visual estimates of the relative intensities of the bands were always recorded. Although such estimates were adequate for many purposes, it was often desirable to have a quantitative estimate of the staining of each band and this was provided by densitometry of the destained gels. Under favourable and carefully-controlled conditions, it may be possible to relate the intensity of staining of a particular band to the amount of that protein applied to the gel, as will be demonstrated later.

For quantitative densitometry, gels were thoroughly destained with 7% (v/v) acetic acid solution to reduce the back-ground staining to the lowest possible level. The stained bands may, however, show a greater tendency to fade if the gels are stored in completely clear acetic acid solution for a long period and so this extra destaining was only carried out after the gels had been measured.

Gels were scanned in a Vitatron Universal Photometer UFD, fitted

with a gel-scanning attachment and a logarithmic/linear chart recorder. The belt drive of the scanner was set to a speed of 1.72cm/min and the chart recorder to a speed of 6cm/min, giving a scan expanded by a factor of 3.488 over life-size. (This is the calculated figure; when checked it was found to be 3.483). The light-source was a mercury-vapour lamp with a collimating slit of width 5mm. In front of this was an adjustable diaphragm and a holder for a green filter (transmission maximum : 500nm). The gel to be scanned was mounted on a clean, scratch-free glass TLC plate (5 x 20cm) and black Perspex strips (7.5cm x 2.0cm x 0.6cm) were positioned either side of the gel. 7%(v/v) acetic acid was then applied from a dropping pipette, to eliminate any air-bubbles. Care was taken to position the Perspex strips parallel to one another and to avoid applying too much acetic acid, since excess solvent on the top surface of the gel considerably increases light-scattering.

The glass plate with the gel on it was placed on the belt drive of the gel-scanner attachment. The belts served to draw the gel, at a constant velocity, through the light beam, over the entrance-slit (8mm x 0.2mm) of the photomultiplier. The adjustable diaphragm was used to set the recorder deflection to zero with a clear section of the (stained) gel positioned in the light-path and the recorder in the logarithmic mode. In this mode, a full-scale deflection (100 divisions) corresponds to an absorbance of 1.0 units. The deflection from the baseline recorded for a given peak was

independent of the zero setting. Noise from the photomultiplier and from light-scattering was generally less than ± 1 scale division (± 0.01 absorbance units) and only rarely exceeded ± 2 scale divisions (± 0.02 absorbance units). The areas under peaks in the traces were measured by the triangulation method, in which tangents are drawn to the ascending and descending limbs of the peaks and the heights of the points of intersection of these tangents, together with the associated lengths for the base-lines, are used to calculate the areas under each peak. The protein concentration in the electrophoretic samples used for the densitometry experiments and in all other samples described in this thesis was measured by the Folin method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as a standard.

Materials.

Unless otherwise stated, all chemicals were purchased from BDH Chemicals Ltd., Poole, Dorset and were of the highest available purity. The standard proteins used for molecular weight calibration were obtained from the following sources: Bovine gamma-globulin (fraction II), serum albumin (fraction V), thyroglobulin, pancreatic ribonuclease-A, trypsin and ovalbumin were purchased from the Sigma Chemical Co., bovine β-lactoglobulin, α-Chymotrypsinogen, pepsin and sperm whale myoglobin were from Koch-Light Laboratories Ltd., and horse-heart cytochrome c was bought from Boehringer Mannheim GmbH.

RESULTS

At the outset of my attempts to isolate and characterise membrane proteins of bovine adrenal chromaffin granules, I made use of the electrophoresis and gel-filtration methods described by Winkler et al. (1970) and Hörtnagl et al. (1971). It was hoped that, in this way, a direct correspondence could be established between new results obtained in Oxford and others emanating from the Innsbruck group.

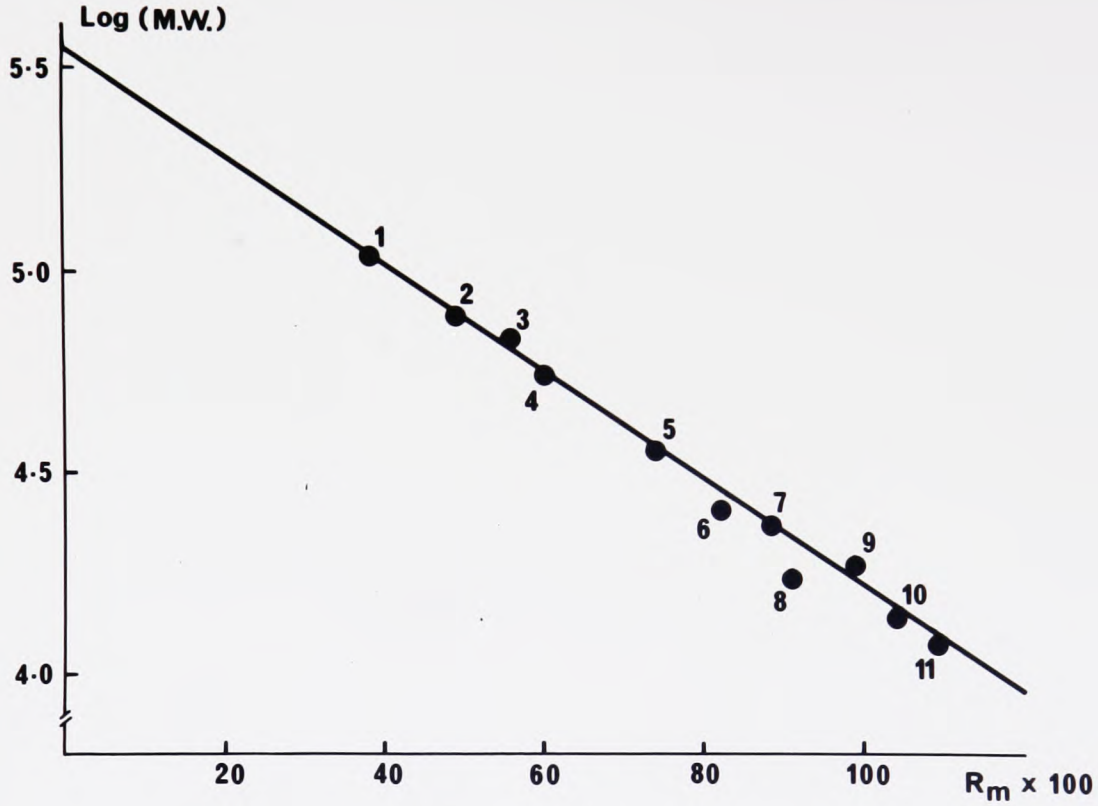
Winkler and co-workers used dilute (3½% acrylamide) polyacrylamide gels, containing 1% SDS, for electrophoretic analysis of the proteins solubilised from washed membranes on treating them with an excess of a solution containing 1% SDS. They "fixed" these gels by immersing them in a quantity of the fixative described by DeVito and Santomé (1966). This fixative solution has a high content (50% v/v) of methanol and soft (dilute) gels shrink considerably when immersed in it. This shrinkage, which is caused by dehydration of the polyacrylamide gel matrix, can result in mechanical damage to the gel surface, even though gels subsequently swell again during destaining with 7% (v/v) aqueous acetic acid. Because of this distortion of the gels during fixation, coupled with the practical difficulty of handling 3½% gels, which are mechanically very weak, and the limited resolution of protein bands achieved when using this system, it was decided that an

alternative electrophoresis method should be developed.

20% aqueous sulphosalicylic acid was found to be an effective fixative and it did not cause distortion problems, but the first major improvement in results was brought about by raising the acrylamide concentration in the gels to 5%, at the same time replacing the sodium borate/1% SDS buffer used by Winkler et al. (1970) by a 0.1M sodium phosphate buffer which contained 0.1% SDS. Under these conditions, 25% aqueous trichloroacetic acid proved to be superior to the fixative of De Vito and Santomé and was therefore adopted routinely, although 50% aqueous trichloroacetic acid was used in some experiments, with equivalent effect.

Molecular Weight Studies.

Even in its simplest form (a single gel of uniform composition and a continuous buffer system) polyacrylamide gel electrophoresis is a powerful analytical tool which may be used to determine the number of proteins present in a sample solution or, in a more quantitative fashion, to estimate the degree of purification achieved by a particular step in the isolation of a protein. Under favourable conditions, electrophoretic analysis is capable of providing direct measurements of molecular properties of individual proteins (Rodbard & Chrambach, 1971) and, in contrast to such techniques as ultracentrifugal sedimentation, it does not require an homogeneous sample. One of the earliest objectives of this project was, therefore, the estimation, by an electrophoretic method similar to


Log (M.W.)
5·5
5·0
4·5
4·0
1
2
3
4
5
6
7
8
9
10
11
20
40
60
80
100
R_m x 100

those described by Shapiro, Vinuela & Maizel (1967) and Weber & Osborn (1969), of the molecular weights of the protein components of bovine adrenal chromaffin granule membranes.

Reduced Proteins.

For this purpose, a series of "standard" globular proteins, of known molecular weights, were dissolved in a solution containing SDS and were reduced with dithiothreitol (final concentration 1%), before being submitted to electrophoresis in 5% polyacrylamide gels. A "calibration graph" was constructed, relating $\log_{10}$ (molecular weight) to relative mobility (see: Fig. 2.1). Figures for the molecular weights of the proteins used to construct this graph were those quoted by Weber & Osborn (1969) except that the molecular weight of the heavy chain of bovine γ-globulin was taken as 55,000. It was found that bovine immunoglobulin-G was not completely dissociated by the conditions used in these experiments and larger fragments were visible in the gels, as well as the expected heavy and light chains. These fragments were used as extra "standard proteins" and fall in the upper end of the molecular weight range.

When molecular weights are to be estimated from measurements of the mobilities of protein bands, it is important that these mobilities should not depend on the treatment, volume or composition of the applied sample. In some experiments, samples were dialysed against a large excess (500ml) of electrolyte buffer, containing dithiothreitol (0.05%). In order to discover whether this prior

Table 2.1

Relative Mobilities and Estimated Molecular Weights of Chromaffin Granule Membrane Proteins after Reduction and Electrophoresis on 5% Polyacrylamide Gels (Molecular Weights Calculated from the Calibration Graph in Figure 2.1).

Mean R_m x 100.	SEM.	No. of Measurements.	Calculated Mean Molecular Weight.	SEM.
5.517	0.039	7	300,527	821
20.262	0.262	11	191,470	1516
30.816	0.623	8	138,766	2694
35.997	0.608	13	118,519	2195
49.193	0.470	12	79,076	1134
54.930	0.454	5	66,297	915
58.205	0.481	13	60,032	883
67.059	0.407	13	45,769	573
75.505	0.554	8	35,349	602
83.838	0.474	10	27,393	402
90.670	0.371	4	22,209	253

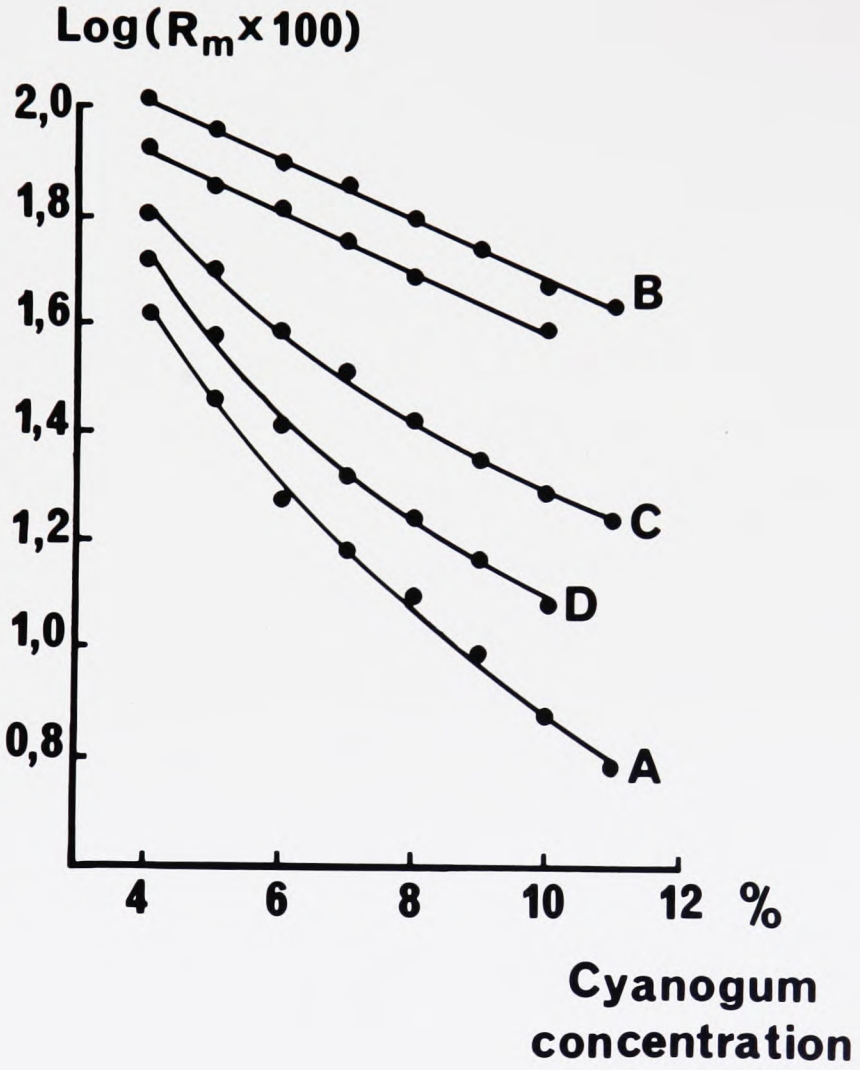
dialysis affected the mobilities of the protein bands, separate calibration graphs were constructed, by least-squares linear regression analysis, for dialysed and undialysed standard protein samples and the slopes of these two lines were compared. The null hypothesis that there is no difference between the slopes may be tested by the "Student's t" method (see: Steel & Torrie, 1960, where the algebraic formula used for this test is given). In the case of the standard proteins no significant difference was found, between the slopes of the two lines, ($p \gg 0.05$) and the data were therefore pooled and the resulting regression equation was used for the calculation of molecular weights.

A comparison of the mean R_m x 100 values for dialysed and undialysed samples of solubilised chromaffin granule membrane proteins revealed a statistically significant difference in only two cases. One of these two components, where a significant difference was shown, is of only moderate staining intensity, but the other is chromomembrin B, one of the major membrane proteins. Both of these bands are of relatively high mobility and it is possible that there may be some fluctuation in the ionic composition of the buffer in the zone immediately following the dye marker band. No distinction has been made between dialysed and undialysed samples when calculating the molecular weights set out in Table 2.1, and it is perhaps surprising that the standard error of the mean molecular weight calculated for chromomembrin B (mean molecular weight 27,393) is

less than 1.5% of the mean value.

One of the consequences of reducing disulphide bonds with dithiothreitol is that multi-chain proteins are split into their constituent polypeptide sub-units. This reduction is a standard feature of published methods for the electrophoretic determination of molecular weights in SDS-containing gels and it has been suggested (Reynolds & Tanford, 1970) that the observations that all reduced proteins bind the same weight ratio of SDS, and that the reduced protein-detergent complexes all adopt the same conformation in solution, may provide a theoretical validation for this practice. However, while reduction may be desirable when single, pure proteins are being studied, it gives rise to overlapping bands when applied to samples of membranes which contain a variety of different proteins. Hörtnagl et al. (1971) have reported that one of the major chromaffin granule membrane components - chromomembrin A - split into two subunits when exposed to β-mercaptoethanol and another, unconfirmed, report (Kirshner & Kirshner, 1969) suggested that chromogranin A - thought to be identical with "component C" in the nomenclature of Winkler et al. (1970) - is also cleaved on reduction, to give two identical subunits of molecular weight ca. 40,000. In view of these reports, it seemed desirable to try to determine whether molecular weights could be obtained for chromaffin granule membrane proteins without reducing them.

All the methods used to determine molecular weights from

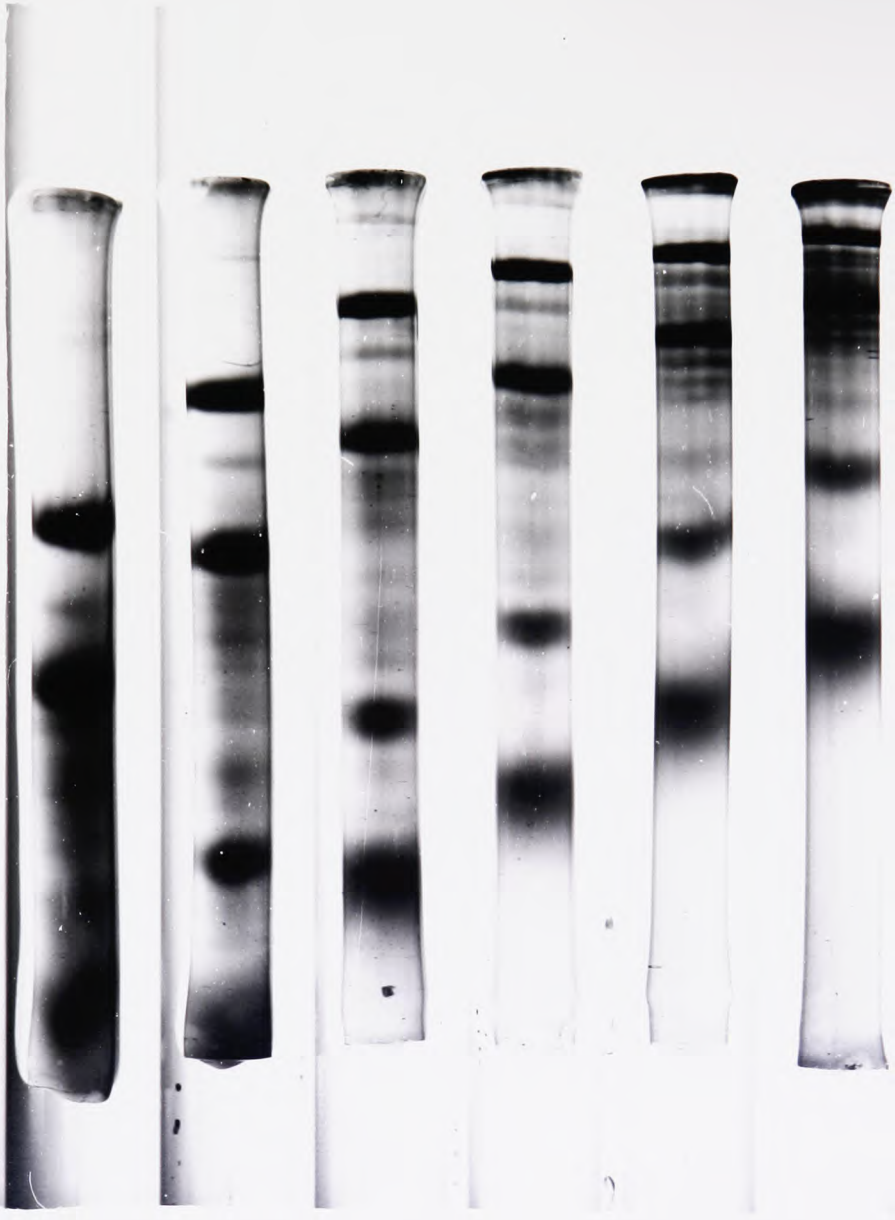

Log(Rm x 100)
2,0
1,8
1,6
1,4
1,2
1,0
0,8
B
C
D
A
4
6
8
10
12
%
Cyanogum
concentration

measurements of electrophoretic mobilities in gel media depend on the validity of the relationship between mobility and gel concentration first described by Ferguson (1964). This relationship, generally called the "Ferguson equation", states that:

$$\text{Log}(M) = \text{Log}(M_o) - K_r T \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where M is the observed mobility of a charged macromolecule at a gel concentration T, M_o is the mobility at zero gel concentration (the "free solution mobility") and K_r is a constant, termed the "retardation coefficient". Plots of the logarithm of the mobility (or relative mobility) of a protein obeying this equation, against gel concentration (T) will therefore be linear and such plots are referred to as "Ferguson plots".

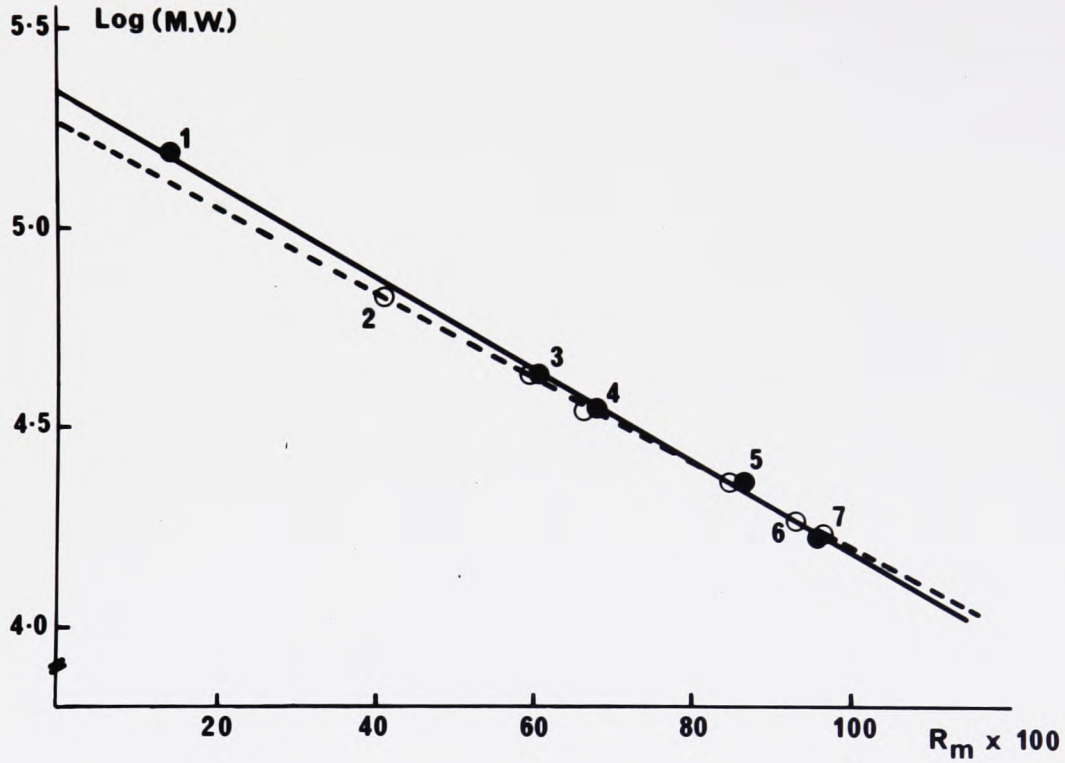
The Ferguson equation has been found to describe the electrophoretic behaviour of (undenatured) proteins in starch (Ferguson, 1964) and polyacrylamide gels (Morris, 1967; Hedrick & Smith, 1968; Rodbard & Chrambach, 1970) and should apply to any charged molecule. Recently, Neville (1971) and Banker and Cotman (1972) have shown that this relationship also holds for reduced protein/SDS complexes derived from a variety of globular proteins commonly used as molecular weight "standards". Clearly, valid molecular weight estimates can only be obtained for unknown proteins when they too obey the Ferguson equation. If they do not, the "molecular weights" observed will be dependent on the concentration



of the gel in which the comparison with the standard proteins was made. There have been several reports (Bretscher, 1971; Segrest, Jackson, Andrews & Marchesi, 1971) that certain erythrocyte membrane glycoproteins exhibit just such "gel-dependent" molecular weights and it was decided to examine unreduced chromaffin granule membrane proteins for evidence of such an effect.

Unreduced Proteins.

One pellet of chromaffin granule membranes was therefore dissolved with 2ml of 2% sodium dodecyl sulphate and the resulting solution was dialysed overnight against 500ml of 0.1M sodium phosphate buffer (pH 6.5), containing 0.1% SDS. After dialysis, the solution appeared slightly turbid and was centrifuged (100,000g_{av} for 30 min) before use, to give a clear solution. In three separate experiments, 25 microlitre aliquots of this solution were applied to gels whose (individual) acrylamide concentrations ranged from 4% to 11%, and electrophoresis was carried out as described earlier. In this way, three estimates of R_m x 100 were obtained for each protein band, at each gel concentration. The results of these experiments are shown in Fig. 2.2 in which the logarithms of the relative mobilities of three major and two minor bands have been plotted against the gel concentration. This plot shows that, while straight lines are obtained for chromomembrin B and one of the two minor components, chromomembrin A, component C and the other minor component do not obey the Ferguson equation. The appearances of the stained bands at


Log (M.W.)
5·5
5·0
4·5
4·0
1
2
3
4
5
6
7
20
40
60
80
100
R_m x 100

each of the various gel concentrations are shown in Plate 2.1, from which it can be seen that all of the bands visible in 4% gels still enter the gel when the acrylamide concentration is raised to 9%. 6% acrylamide gels were used in all subsequent experiments, unless stated otherwise, as gels of this concentration were found to give optimum resolution of the bands.

Although it would only be possible to obtain valid molecular weights for some, rather than all, of the chromaffin granule membrane proteins, a calibration graph was constructed at this acrylamide concentration (6%), using standard globular proteins that had been denatured with SDS but not reduced. Even without reduction of the standards, there was a linear relationship between the logarithm of the molecular weight and the relative mobility (correlation coefficient $r = -0.9981$) for all of the standard proteins examined, except bovine serum albumin. This protein has been noted to have an anomalously high mobility when not reduced (Fairbanks, Steck & Wallach, 1971) and, since the same observation was made in the present studies, points for this protein were not included when calculating the regression equation for the calibration graph. When this (unreduced standard) calibration graph was compared with another (Fig. 2.3), derived from experiments in which the standard proteins had been reduced with an excess of dithiothreitol, no statistically significant difference was found between the slopes of the two graphs. The correlation coefficient in this latter case (reduced standards) was

Table 2.2

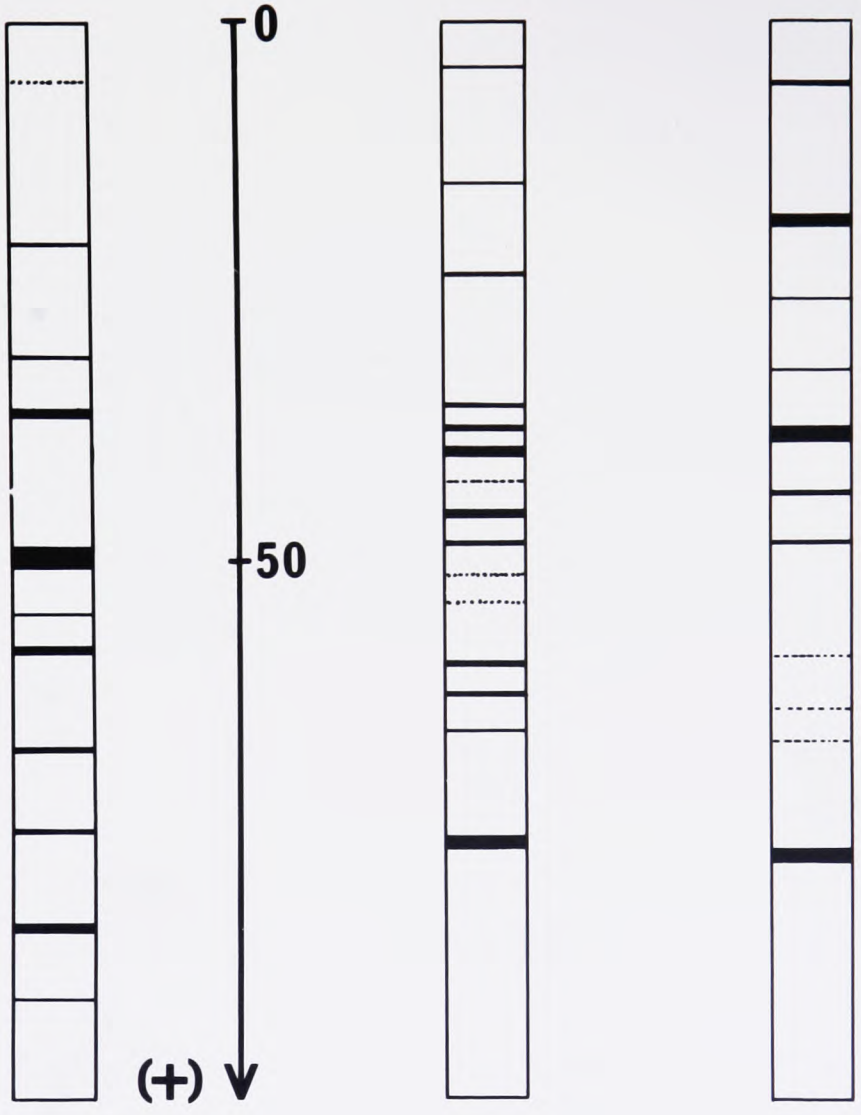
Relative Mobilities and Estimated Molecular Weights of Unreduced Chromaffin Granule Membrane Proteins submitted to Electrophoresis in 6% Polyacrylamide Gels (Calibration graph: Fig. 2.3)

Mean R_M x 100.	No. of Determinations.	S.D.	S.E.M.	Calculated Mean Molecular Weight.	S.E.M.
5.792	13	0.967	0.268	189,537	1,331
18.510	13	0.977	0.271	135,500	975
25.719	12	0.973	0.281	112,024	837
32.370	9	1.211	0.404	94,005	1,005
38.329	13	1.049	0.291	80,319	626
43.777	13	0.971	0.269	69,558	498
48.583	10	1.078	0.341	61,276	556
58.702	11	0.987	0.298	46,913	369
63.678	9	0.912	0.304	41,138	329
66.789	7	0.919	0.347	37,895	345
77.306	13	1.631	0.452	28,728	339

-0.9984 and it was concluded that either graph could justifiably be used when calculating molecular weights, although the "unreduced standards" graph was preferred when the unknown proteins were unreduced.

Relative mobility measurements from a number of different electrophoretic experiments in which unreduced chromaffin granule membrane proteins were analysed in 6% polyacrylamide gels are tabulated in Table 2.2, along with the mean molecular weights derived from the (unreduced standards) calibration graph. Individual molecular weight estimates, calculated from each of the relative mobility measurements, were pooled to obtain the mean molecular weights in this table. It is apparent from these data that the observed variances (= square of standard deviation) of the relative mobility values were virtually constant throughout the range 10 - 100 R_m x 100 units (mean of the standard deviations = 1.06). This was also found to be true for the standard proteins and is an important result, since an unweighted regression equation was used to construct the calibration graphs (Fig. 2.3) and this equation makes the assumption that all observations are subject to the same degree of error.

In a smaller number of experiments, the membrane proteins were reduced with dithiothreitol before being analysed on 6% gels. Mean molecular weights were calculated for each of the components, this time using the "reduced standards" calibration graph. The


0
50
(+)

band patterns observed in these experiments are illustrated in Fig. 2.4, in which typical patterns for the unreduced membrane proteins in 6% gels and for dithiothreitol-reduced membrane proteins in 5% gels have also been included. Better resolution of the bands was obtained in 6% gels, compared with the earlier experiments in 5% gels, and a number of minor bands could be seen which were not easily distinguished in the more dilute gels.

More importantly, the single, broad, intensely-staining band (estimated molecular weight 79,076) of intermediate mobility seen in 5% gels appeared to consist of two, and at lower loadings occasionally three, bands in the 6% gels, although these intense bands were not completely resolved from one another. Both chromogranin A and, as will be shown later, chromomembrin A give rise to bands in this region of the gel when reduced and it was not possible to identify with confidence the origins of the bands when both proteins were present in the sample, as was the case when SDS-extracts of whole chromaffin granule membranes were examined.

The molecular weights calculated from these experiments in 6% gels were in reasonable agreement, below ca. 100,000 daltons, with those found in 5% gels (see Table 2.3). Above this limit, the agreement is less good, although this may partly be explained by the increasing effect of experimental error.

Soluble Chromaffin Granule Proteins.

It was apparent from the studies just described that the "washed

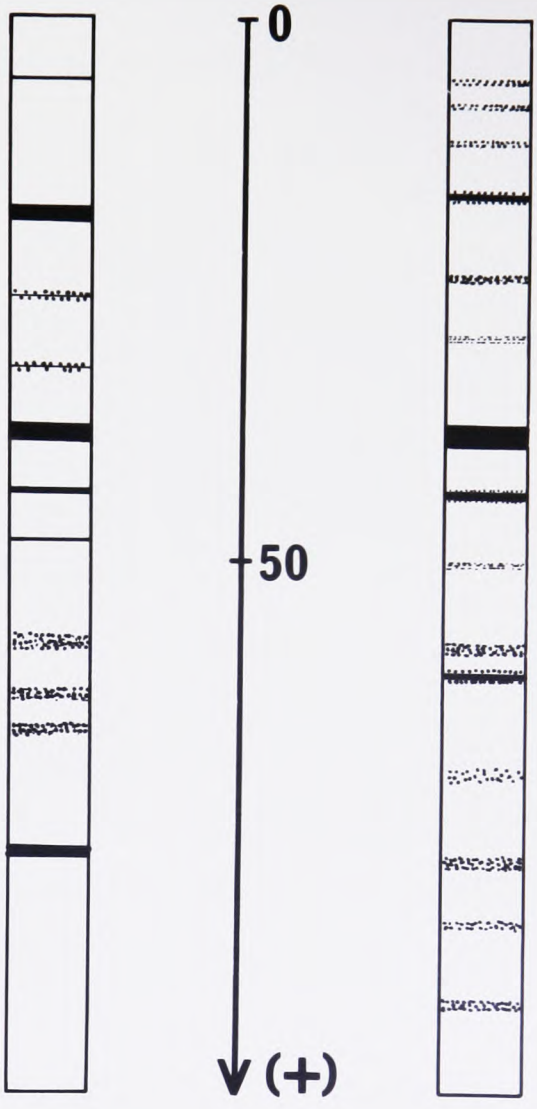
Table 2.3

Mean Molecular Weights and Staining Intensities of Chromaffin Granule Membrane Proteins.

5% Gels (reduced)		6% Gels (reduced)		6% Gels (unreduced)	
Molecular Weight	Staining	Molecular Weight	Staining	Molecular Weight	Staining
300,527	v. faint	-		-	
191,470	faint	165,973	faint	189,537	faint
138,766	faint	128,649	faint	135,500	strong
118,519	medium	104,706	faint	112,024	faint
-		-		94,005	faint
79,076	strong	77,377	strong	80,319	strong
-		73,543	strong	-	
-		69,291	strong	69,558	medium
66,297	faint	65,311	v. faint	-	
60,032	medium	60,378	medium	61,276	faint
-		56,619	faint	-	
-		52,795	v. faint	-	
-		49,639	v. faint	-	
45,769	medium	43,354	medium	46,913	faint
-		40,279	faint	41,138	v. faint
35,349	medium	37,073	faint	37,895	v.faint
27,393	strong	28,485	strong	28,728	strong
22,209	faint	-		-	

membrane" preparation, referred to as "chromaffin granule membranes", contained a relatively large number of polypeptide chains, which could be distinguished from one another in the SDS/6% polyacrylamide gel system, most of them falling into the category of "minor components". It seemed probable, therefore, that some traces of soluble chromaffin granule proteins might still be adhering to the membranes, perhaps giving rise to some of the minor components. In the earliest paper describing this membrane preparation, Winkler et al. (1970) suggest that component C represents a small amount of contaminating chromogranin A, estimated immunochemically to represent ca. 1% of the total protein. In order to identify the adsorbed soluble proteins, and thus show more clearly whether there are proteins that may be considered to be truly specific for the membrane phase, a number of experiments were performed in which aliquots of dialysed "soluble lysate" (see earlier, under Methods) were incubated with excess SDS and then submitted to electrophoresis in 6% gels, in the same way as for the solubilised membranes.

The, entirely unexpected, results of these experiments are shown diagrammatically in Fig. 2.5, in which the stained pattern typical of soluble lysate proteins is compared with that given by unreduced membranes. (See also Plate 2.2 for a comparison of typical patterns of unreduced and reduced membrane proteins and unreduced soluble lysate proteins, all run in 6% polyacrylamide gels). Surprisingly, almost all of the components visible in the pattern

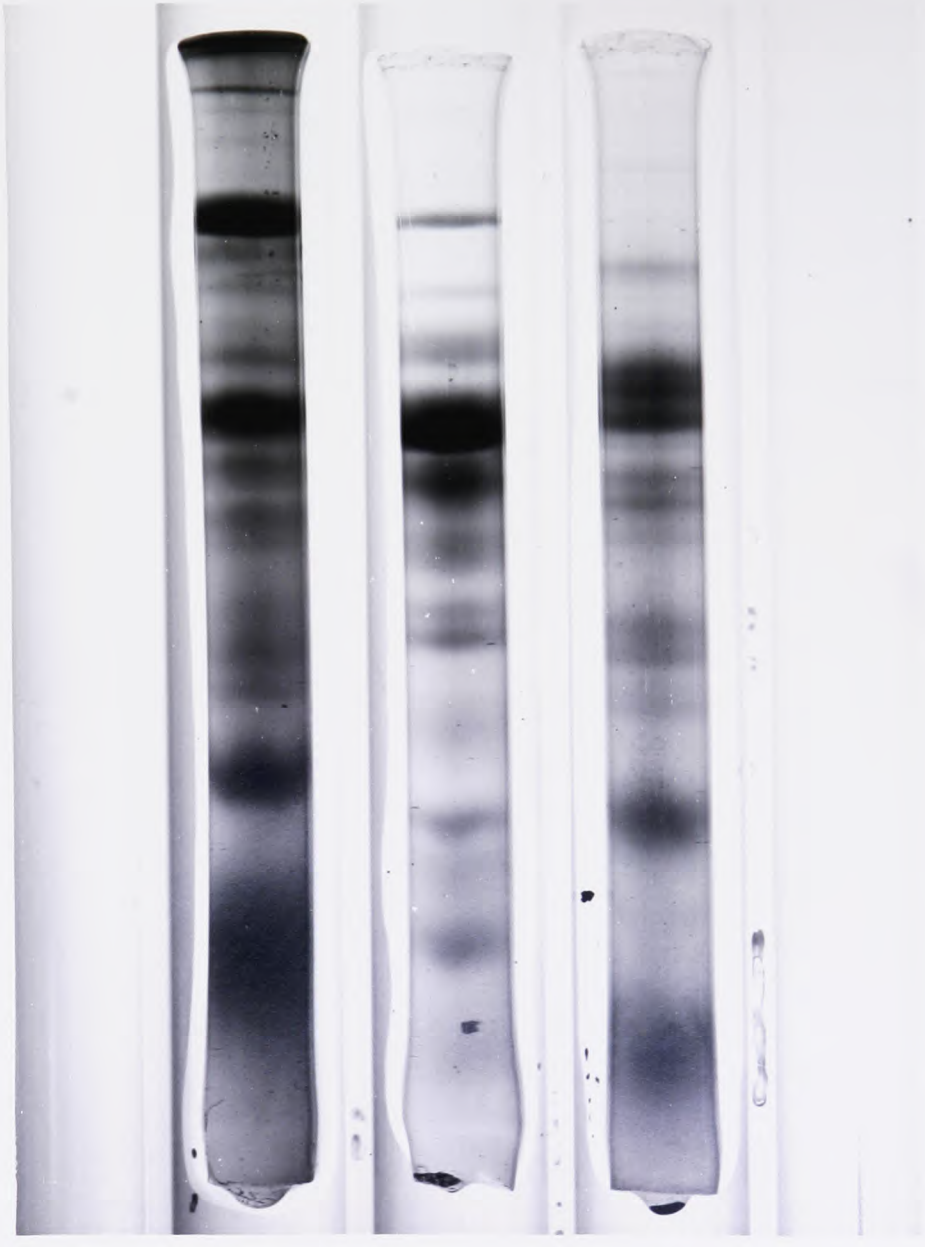

0
50
(+)

given by unreduced membranes are also evident in the soluble lysate pattern: for the most part with similar relative staining intensities.

Quantitative Densitometry of Chromogranin A.

Since this experiment suggested that the chromaffin granule membranes might still contain appreciable amounts of protein from the soluble phase of the granule and since there is disagreement between different investigators on this point, Winkler et al., (1970) reporting that chromogranin A accounted for 4% of the protein in the membrane pellet whereas Helle and co-workers have found it to represent 73 -75% (Helle & Serck-Hanssen, 1969), 15 - 35% (Helle, 1971) and 21% (Helle, Lagercrantz & Stjärne, 1971) of the membrane protein, I decided to try to measure the chromogranin A content of my membrane pellet by an independent (non-immunological) method, for which quantitative densitometry was the best available alternative.

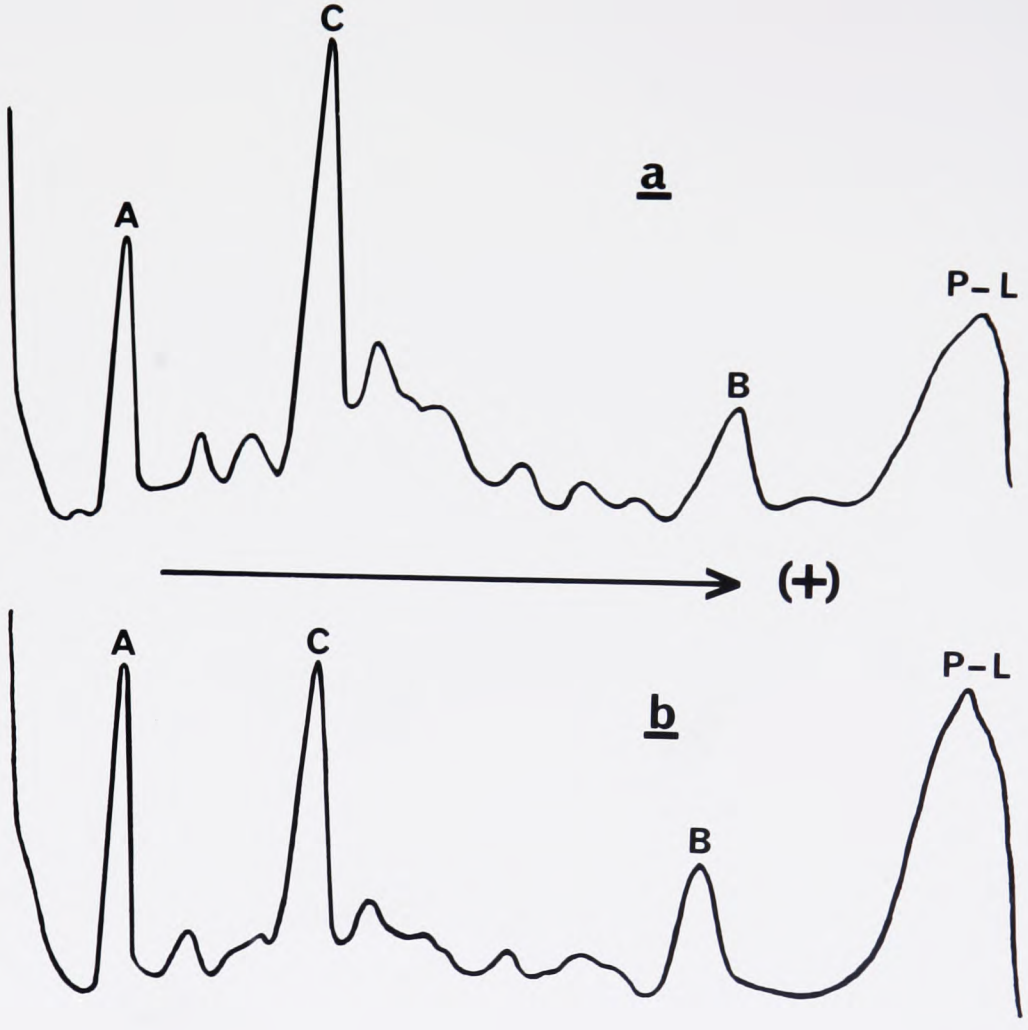
Accordingly, I prepared a solution of highly-purified chromogranin A, by the ion-exchange method described under "Methods", measured the protein concentration and, after dilution with a known volume of 0.01M sodium phosphate buffer containing SDS and sucrose, submitted aliquots of known volume to electrophoresis with the sodium phosphate/SDS buffer system. After fixation and staining, the gels were scanned in a Vitatron densitometer fitted with a green filter and the peak areas of the traces were measured. It was found that the peak area was linearly proportional to the chromogranin A content of the sample over the range 0 - 12μg of chromogranin A (correlation coefficient



for least-squares fit of the data = 0.9974).

The chromogranin A content of washed membranes was then determined by carrying out the electrophoresis/staining/densitometry with aliquots of an SDS-extract of chromaffin granule membrane pellet of known (total) protein concentration. Measurements on three aliquots of the same SDS-extract of membranes, containing 10, 20 and 30 μg of total protein respectively, showed that chromogranin A accounted for 13.0%, 13.5%, 12.7% (Mean = 13.1%) of the total membrane protein. In a sample of membranes from the same batch of chromaffin granules but lysed by the dialysis procedure of Helle (1971) and analysed in the same experiment, chromogranin A was found to make up 18.2% (mean of three determinations) of the total protein. Representative densitometer traces from these two samples are shown in Fig. 2.6, for comparative purposes.

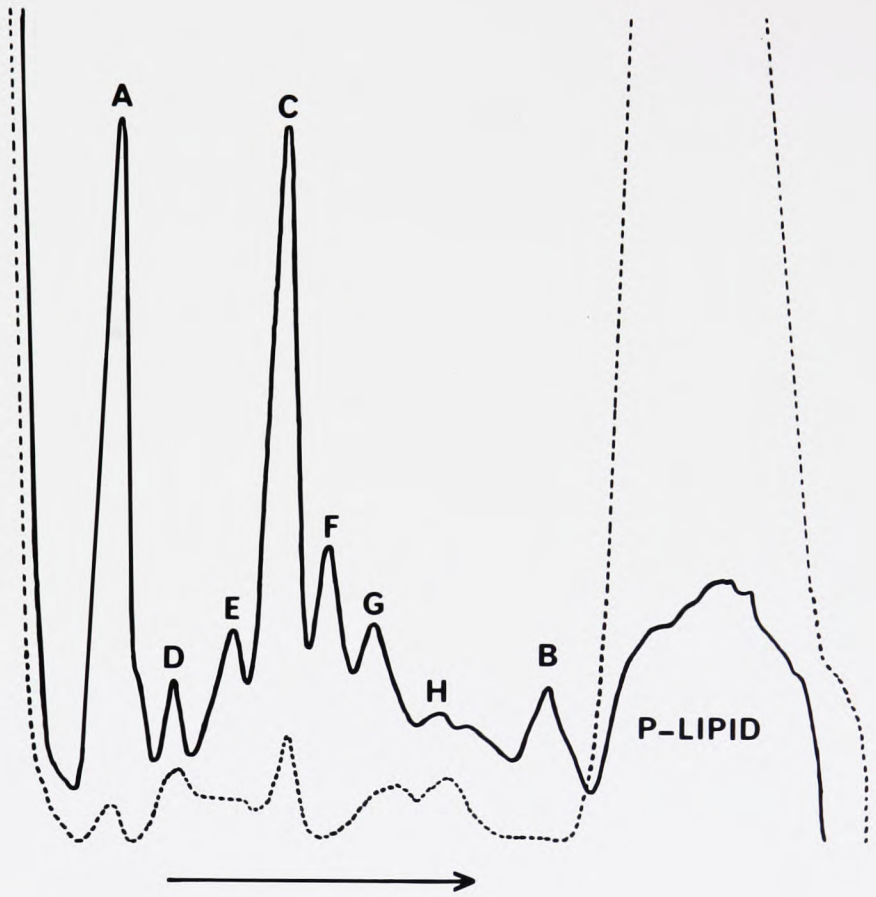
Other factors, apart from contamination by soluble proteins, could contribute to increasing the number of bands observed in the patterns given by membrane samples; for example, some proteins are only dissociated into their constituent subunits by extreme treatment, while others may be particularly susceptible to proteolysis by lysosomal peptidases, either during isolation or storage. It was established experimentally that the mobilities and staining intensities of the membrane proteins were not dependent on the length of storage at -20°C prior to solubilisation. Similarly, omitting the bromophenol blue tracker dye, raising the SDS concentration and heating the sample in boiling water for five minutes during the


C
a
A
P-L
B
(+)
A
C
P-L
b
B

solubilisation were all without effect. It was also found that including 5M urea in the solubilising solution, either in the presence or absence of dithiothreitol, did not affect the band-patterns obtained with a preparation of microsomal membranes (prepared by high-speed centrifugation of the "medium speed supernatant" remaining after isolation of the "large granule fraction" from homogenates of bovine adrenal medullae) which were markedly more difficult to dissolve with SDS than chromaffin granule membranes. For these reasons, it seems likely that the bands observed in the gels of chromaffin granule membranes represent individual protein components, rather than aggregates or break-down products. It is, however, possible that more than one distinct polypeptide chain may contribute to the staining intensity of any particular band and fortuitous overlaps of this kind cannot be excluded, although the similarity of the patterns observed in 5% and 6% gels suggests that such overlaps are not common.

Membrane Glycoproteins.

It was remarked earlier that the major glycoprotein component of erythrocyte membranes showed anomalous electrophoretic behaviour in polyacrylamide gels and the observation of similar, atypical behaviour of certain proteins from chromaffin granule membranes (Fig. 2.2) raised the question of whether these proteins were also glycoproteins. Accordingly, in some experiments gels were treated, after fixation, with periodic acid and Schiff's reagent (see: Methods) to visualise carbohydrate-containing material. Several


A
C
F
E
G
D
H
B
P-LIPID

bands were observed to be stained by this procedure and Fig. 2.7 shows a densitometric scan of a gel which had been treated in this way (broken line), compared with a scan of a similar gel which had been stained with Amido Black in the normal manner (continuous line). It is immediately evident that the bands that are stained by the periodate/Schiff method are those which display anomalous electrophoretic properties; and that chromomembrin B, which gives a linear Ferguson plot, does not appear to be stained. (The intense reaction at the bottom end of the gel is caused by the lipid components of the chromaffin granule membrane, which migrate with, or slightly ahead of, the dye). In the case of component C (= chromogranin A) and chromomembrin A (= dopamine β-hydroxylase), the finding of a carbohydrate-positive reaction obtained with Schiff's reagent has been complemented by direct chemical analyses which indicate the presence of carbohydrate (Smith & Winkler, 1967b and H. Hörtnagl, unpublished data).

Both chromaffin granule membranes and the soluble granule proteins contain a small amount (ca. 3%w/w) of sialic acids, chiefly N-glycollyl-neuraminic acid (S.F. Bartlett, unpublished observations, 1970) and, to try to show whether the negative charges introduced by this carbohydrate moiety contribute to the deviations from linearity in the Ferguson plots, an aliquot of a suspension of chromaffin granule membranes was exhaustively digested with V. Cholera neuraminidase (Sigma Chemical Co.), prior to solubilisation with SDS

and electrophoresis. Although this digestion released 67% of the total content of sialic acid, as measured by the 2-thiobarbituric acid method (Warren, 1959), the relative mobilities of the various protein bands were unaffected.

An Alternative Approach for Calculating Molecular Weights.

Since Shapiro et al. (1967), Weber & Osborn (1969) and Dunker & Rückert (1969) reported that a linear relationship is observed between the logarithm of the molecular weights of a series of "standard" proteins and their relative mobilities on electrophoresis in SDS-containing polyacrylamide gels, it has become accepted practice to estimate the molecular weights of unknown proteins from a calibration graph of this kind, and this procedure was employed to calculate the molecular weights for chromaffin granule proteins listed earlier (Tables 2.1, 2.2 and 2.3). While the originators of this approach made clear the fact that the linear relationship between the logarithm of molecular weight and relative mobility was established empirically, subsequent authors have usually chosen to regard this as being unimportant and have accepted the validity of the method without comment.

However, Neville (1971) has shown, both theoretically and experimentally, that the relationship is sigmoidal, with an approximately linear portion in the intermediate mobility range (which is generally the region of most interest, corresponding to molecular weights less than ca. 100,000 and greater than 20,000).

Table 2.4

Molecular Weight Estimates for Unreduced Chromaffin Granule Membrane Proteins from 6% Gels.

		From Log(MW) vs. R_m x 100.		From Log (R_m x 100) vs. MW.	
Mean R_m x 100	No. of Measurements.	Mean Mol. Wt.	Std. Error.	Mean Mol. Wt.	Std. Error.
5.792	13	189,537	1331	222,727	3315
18.510	13	135,500	975	135,504	1097
25.719	12	112,024	837	110,991	823
32.370	9	94,005	1005	93,882	932
38.329	13	80,319	626	81,293	574
43.777	13	69,558	498	71,397	461
48.583	10	61,276	556	63,652	526
58.702	11	46,913	369	49,569	378
63.678	9	41,138	329	43,514	354
66.789	7	37,895	345	39,966	386
77.306	13	28,728	339	29,097	433

He demonstrated that complexes of SDS with standard proteins gave linear Ferguson plots, with a common extrapolation point at a gel concentration of 0% acrylamide, although proteins of molecular weight greater than 60,000 showed some deviation from linearity at gel concentrations greater than 11%. He also showed that plots of the logarithm of relative mobility versus molecular weight were linear over a wide range of molecular weights and that this observation could be justified on theoretical grounds. This work was confirmed and extended by Banker &Cotman (1972), who showed that both the retardation coefficients (K_r) and the (extrapolated) "free" relative mobilities (R_{m_0}) of SDS/protein complexes were linearly related to their molecular weights.

Following the conclusions of these two studies, the data for the "standard" calibrating proteins were recalculated, to give plots of log (relative mobility) versus molecular weight. For unreduced standards on 6% polyacrylamide gels, the correlation coefficient was -0.9970 and this may be compared with the value of -0.9981 from the plot of log (molecular weight) versus relative mobility. However, this still indicates a very highly significant degree of correlation ($p < 0.001$) and the experimental data for chromaffin granule proteins were re-examined, to give estimates of molecular weight by this alternative method. As an illustration of the results, molecular weight estimates obtained by each procedure are set out in Table 2.4, for unreduced chromaffin granule membrane proteins, run in 6% gels.

The values obtained from the plot of log(R_m x 100) versus molecular weight were comparable to, but in general slightly larger than, the values calculated from the more commonly-accepted graph of log (molecular weight) versus relative mobility, and this was also found to be the case when both the standards and the membrane proteins were reduced with dithiothreitol and run in 6% gels. The correspondence between the two methods was less satisfactory for the measurements on 5% gels, but the errors in the observations were greater in these experiments.

Reduction of Dopamine β-hydroxylase/chromomembrin A.

It was remarked earlier that treatment of SDS-extracts of entire chromaffin granule membranes with the disulphide-bond reducing agent dithiothreitol increased the number of electrophoretically-distinguishable components and led to the overlapping of certain bands. The most striking change (see: Plate 2.2) was seen with chromomembrin A, and the strong band at R_m x 100 = 18.5 characteristic of this protein disappeared on reduction, there being a concomitant increase in the staining in the region of R_m x 100 = 35. Hörtnagl et al. (1971), working with samples of chromomembrin A purified by their borate/SDS/G-200 column method, had noted that this protein was split into two subunits by β-mercaptoethanol and they found that the more strongly-staining of these two subunits, which they named "chromomembrin A_1", had a similar mobility to component C (which is chromogranin A), on electrophoresis in SDS-containing gels.

When I had prepared some rechromatographed chromomembrin A (see next chapter for details of the method used), I decided to try to estimate the molecular weights of these two subunits electrophoretically. After reduction with a large excess of dithiothreitol, my preparation gave a very intensely-staining band at ca. $R_m \times 100 = 36$ but only a very faint faster-running band ($R_m \times 100 =$ ca. 47). An additional component at $R_m \times 100 =$ ca. 26 appeared in gels loaded with both unreduced and reduced chromomembrin A and did not appear to be affected by reduction. This component is labelled "D" in Fig. 2.7 and was the principal contaminant of the rechromatographed chromomembrin A. Although Hörtnagl et al. (1971) had reported that "chromomembrin A_2" (the other subunit) was not intensely-stained, the component at $R_m \times 100 = 47$ was so faint compared with chromomembrin A_1 in my gels that it began to seem doubtful whether this band did, in fact, represent a subunit of chromomembrin A and was not simply a contaminant, since the pattern from unreduced chromaffin granule membranes also included a component at $R_m \times 100 = 48.6$ (see Table 2.2).

Hörtnagl, Winkler & Lochs (1972) have shown that enzymically active dopamine β-hydroxylase isolated from soluble lysate, by cetavlon-precipitation of the chromogranins, has a similar amino acid composition and electrophoretic mobility (in two different gel systems) to chromomembrin A, and they concluded that the two were identical. Although it did not prove possible, in my experience,

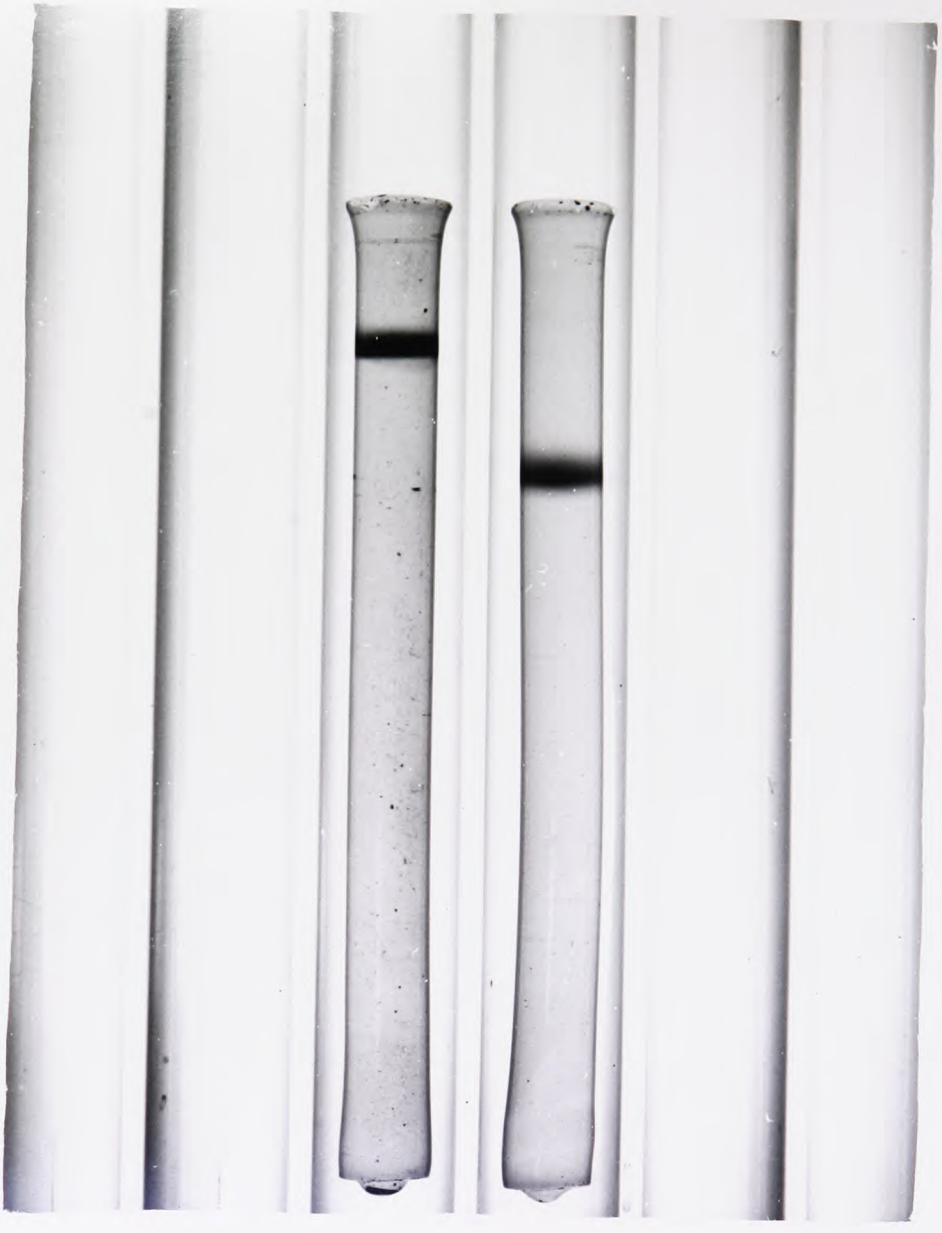


Plate 2.3. The Electrophoretic Pattern of Dopamine β-hydroxylase Before and After Reduction with Dithiothreitol.

The samples were (left) unreduced and (right) reduced dopamine β-hydroxylase, isolated by the ion-exchange method and run in 6% gels.

to obtain a homogeneous preparation of chromomembrin A, the ion-exchange method (described earlier) did give an absolutely pure preparation of soluble dopamine β-hydroxylase. When this was treated with dithiothreitol and submitted to electrophoresis in phosphate/SDS gels, only one component was seen (see: Plate 2.3), and this component had a relative mobility of $R_m \times 100 = 36$. I therefore conclude that the faster-running band seen in gels from the reduced chromomembrin A preparation is a contaminating protein and that, by inference, "chromomembrin A_2" is not a true subunit of chromomembrin A.

CHAPTER 3.

Gel-Filtration of SDS-dissolved Chromaffin Granule Membrane Proteins.

Page

Methods

Gel-filtration of SDS-dissolved membrane proteins 56

Results

Column Chromatography of dissolved chromaffin granule membrane proteins. 60

Sephadex G-200 chromatography 60

Chromatography on Sepharose 4B 62

Chromatography on Sepharose 6B 63

Preparative-scale chromatography on Sepharose 6B 65

The nature of the material in the final peak 69

Further purification of individual proteins by rechromatography 70

Rechromatography of Fraction A 71

Rechromatography of Fraction B 72

Rechromatography of Fraction C 74

Estimation of molecular weights by chromatography 76

METHODS

Gel-filtration of SDS-dissolved Membrane Proteins.

A tris-hydrochloric acid buffer containing 0.1% SDS was used for most gel-filtration experiments, and a concentrated stock solution of this buffer was made as follows. 121.14g of ARISTAR tris (free base), 20.0g of sodium dodecyl sulphate ("Specially pure: for biochemical research") and 4.0g of sodium azide were dissolved in ca. 700ml of distilled water. This solution was then titrated to pH 7.85, by careful addition of ARISTAR concentrated hydrochloric acid, and was then made up to a volume of 1 litre. A considerable amount of heat was liberated during the neutralisation process and it was usually necessary to cool the solution before making the final pH-adjustment, before making up to 1 litre. This stock solution was diluted 1 + 19 (v/v) with distilled water to give column eluant, which had a composition: 0.05M tris, 0.1% SDS, 0.02% sodium azide and a final pH of 7.75 at 22°. In some early experiments, a column buffer containing 1.0% SDS in sodium borate (pH 9.6, 0.0125M in borate ion) was used, as recommended by Hörtnagl et al., (1971) and this was made as described in "Data for Biochemical Research" (2nd Edition, 1969, Eds. Dawson, R.M.C., Elliott, D.C., Elliott, W.H. & Jones, K.M., Oxford University Press, p.497), based on the formulation of Bates & Bower (1956).

Sephadex G-200 was swollen in distilled water by the rapid

method (heating in a bath of boiling water for 5 hours). Sephadex G-200, Sepharose 4B and Sepharose 6B were equilibrated with column buffer by repeated shaking with aliquots of buffer, allowing the gel to settle and then sucking off and discarding the supernatant buffer. This process, which was carried out over the course of 1-2 weeks, using several litres of buffer, resulted in the removal of a portion of the "fines" but the gel beads were not specially fractionated according to particle size. After equilibration, the gel beads were suspended in column buffer and packed into Perspex columns (Wright Scientific Ltd., 3, Lower Road, Kenley, Surrey, CR2 5NH), by pumping downwards at a flow-rate which was twice the maximum flow-rate to be employed during separations. When the bed-height had stabilised pumping was stopped and the filler-tube was quickly removed from the top of the column and replaced by an end-cap. Pumping was then resumed in the reverse direction, i.e.: from bottom to top, at the rate to be used for subsequent chromatographic separations. A volume of buffer equal to at least twice the total volume of the column was pumped through each column before carrying out any chromatographic separation and a similar volume was pumped through between individual chromatographic "runs".

For sample application, the reservoir containing the column eluant was disconnected from the inlet-tubing of the peristaltic pump, a quantity of sample (not exceeding 1.5% of the total column volume) was pumped into the column (at the bottom) and the buffer

supply was then re-connected. A (measured) volume of effluent from the top of the column was then run to waste before fraction-collecting was begun. Fractions were collected with an LKB Ultrorac fraction-collector, using the drop-counting mode, and average fraction-volumes were:- 4 x 60 column: 2.56ml, 8 x 90 column: 2.92ml and 16 x 90 column: 5.00ml. The separations were carried out at room temperature, since SDS is only sparingly soluble in the cold.

To gauge the degree of separation achieved by gel-filtration, the absorbances of selected fractions were determined at 280nm and a graph was constructed, showing absorbance versus fraction-number. Polyacrylamide gel electrophoresis, with the sodium phosphate/SDS buffer system described in the last chapter, was used to identify the individual proteins comprising each of the peaks appearing in the "elution-profile" graph. Bovine adrenal chromaffin granules contain a b-type cytochrome (Ichikawa & Yamano, 1965; Banks, 1965) with an absorbance maximum at ca. 405nm in SDS-containing buffers, and the elution position of this protein could be determined by direct spectrophotometry. Similarly, the elution position of Blue Dextran 2000 (Pharmacia Ltd.), used to measure the void volume (V_o) of the column, could be determined by measuring the absorbance of the fractions at 600nm, or (more sensitively, but non-specifically) at 280nm. Eluted phospholipids were determined chemically, by measuring the inorganic phosphate remaining after charring aliquots of the eluate at 150° with sulphuric acid, followed by oxidation with hydrogen

peroxide under the same conditions, as described by Bartlett (1959). D-glucose, used as a marker for the inclusion volume of the column, was determined in the eluate by a quantitative anthrone reaction (see: R.G. Spiro in "Methods in Enzymology", Eds. Colowick, S.P. & Kaplan, N.O. Vol. VIII, p.5 for the method used, which is based on that of Roe, 1955).

RESULTS

Column Chromatography of Dissolved Chromaffin Granule Membrane Proteins.

Having shown (Winkler et al., 1970) that chromaffin granule membranes contain a relatively small number of proteins, which could be distinguished from one another by SDS/polyacrylamide gel electrophoresis, the Innsbruck group went on to describe a column chromatographic method for isolating the proteins in milligramme quantities (Hörtnagl et al., 1971). This process involved dissolving the membranes in a sodium borate buffer (pH 9.6) which contained 1% w/v SDS, the resulting solution being chromatographed on a column (118cm x 2.3cm diameter) packed with Sephadex G-200 and eluted at room temperature with the same borate/SDS buffer. Hörtnagl et al., (1971) showed photographs of the electrophoretic patterns obtained from samples of chromomembrin A ("componentA") and chromomembrin B ("component B"), prepared by this column method, which seemed to indicate that the preparations thus produced were homogeneous, and they noted that "the small second peak contains component B, which seems to be well purified". Since I intended to try to carry out chemical investigations of the two major membrane proteins, I chose initially, to use the method for their purification published by the Innsbruck group.

Sephadex G-200 Chromatography.

A large plastic column (90cm x 16cm^2 cross-sectional area) was packed with Sephadex G-200 equilibrated with borate/SDS buffer, an

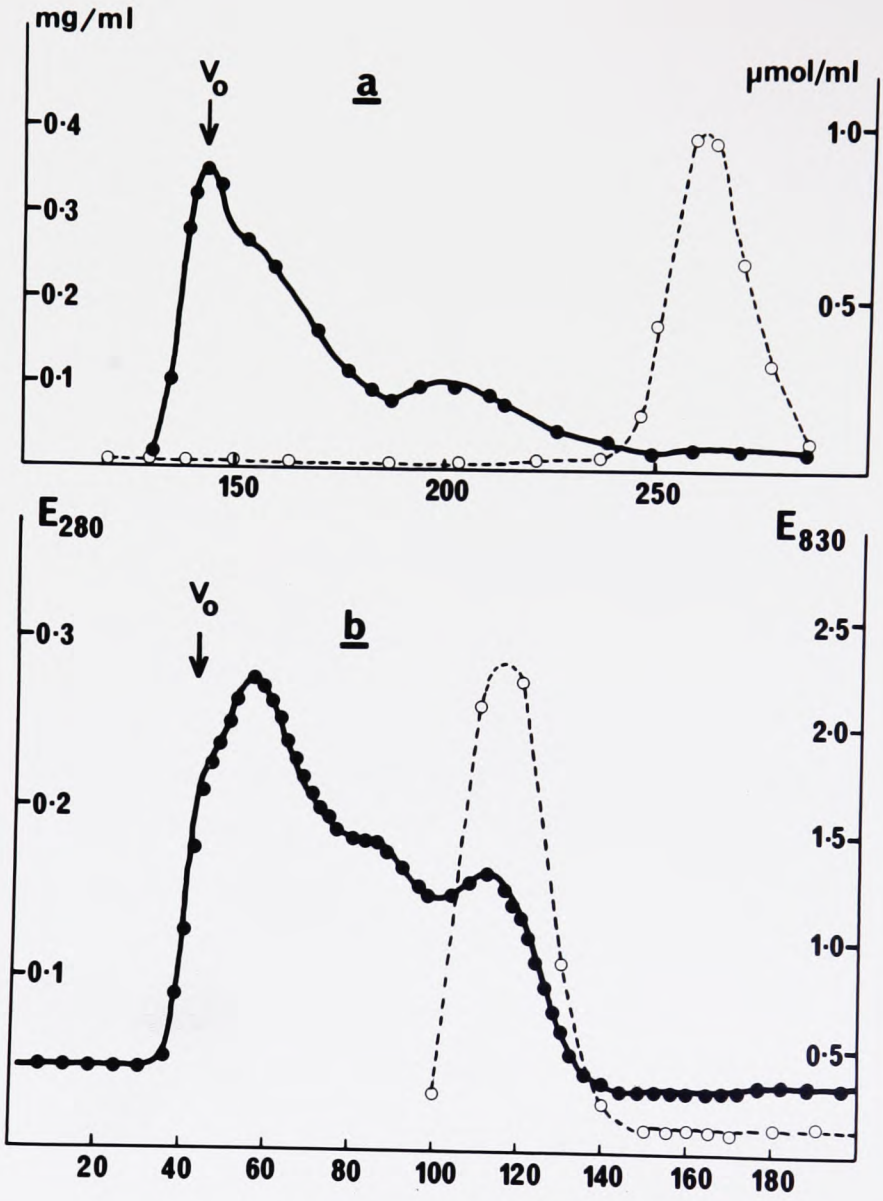


Fig. 3.1. Gel-filtration of SDS-dissolved Chromaffin Granule Membranes on Columns Packed with Sephadex G-200.

a) Data redrawn from Hörtnagl et al., (1971).

b) Elution profile from 90cm x 16cm^2 column.

The elution profile of the phospholipids is shown by a broken line in both figures and the units of the abscissa are millilitres in (a) and fraction number in (b).

described earlier (see under "Methods"). A quantity of chromaffin granule membranes were solubilised as described by Hörtnagl et al., (1971), with the exception that no lipid-extraction was performed prior to treatment with the borate/SDS buffer, and the solution was dialysed overnight at room temperature against a large excess of column eluant buffer before being centrifuged at room temperature (rotor 50Ti, 59,360 g_{av} for 60 mins) and applied to the column. The elution profile obtained is shown in Fig. 3.1, in which the profile reported by Hörtnagl et al. (1971) has been included for comparison (Fig. 3.1a).

Although my elution profile is broadly similar to that reported by Hörtnagl et al. (1971), there are important differences. Like the Innsbruck workers, I found that the bulk of the protein (as judged from its absorption at 280nm) was eluted shortly after the void volume of the column (V_o, established in a separate experiment by chromatographing a sample of Blue Dextran 2000), but this main peak had a higher extinction than the void-volume peak and was followed by a pronounced shoulder. The second major peak was not entirely resolved from this "shoulder". Hörtnagl et al. (1971) did not centrifuge the solution they applied to their column and it is possible that some of the protein content of their void-volume peak may be derived from undissolved membrane fragments. As will be shown later, a small amount of very high molecular weight material remains even after centrifugation. No more ultraviolet-absorbing

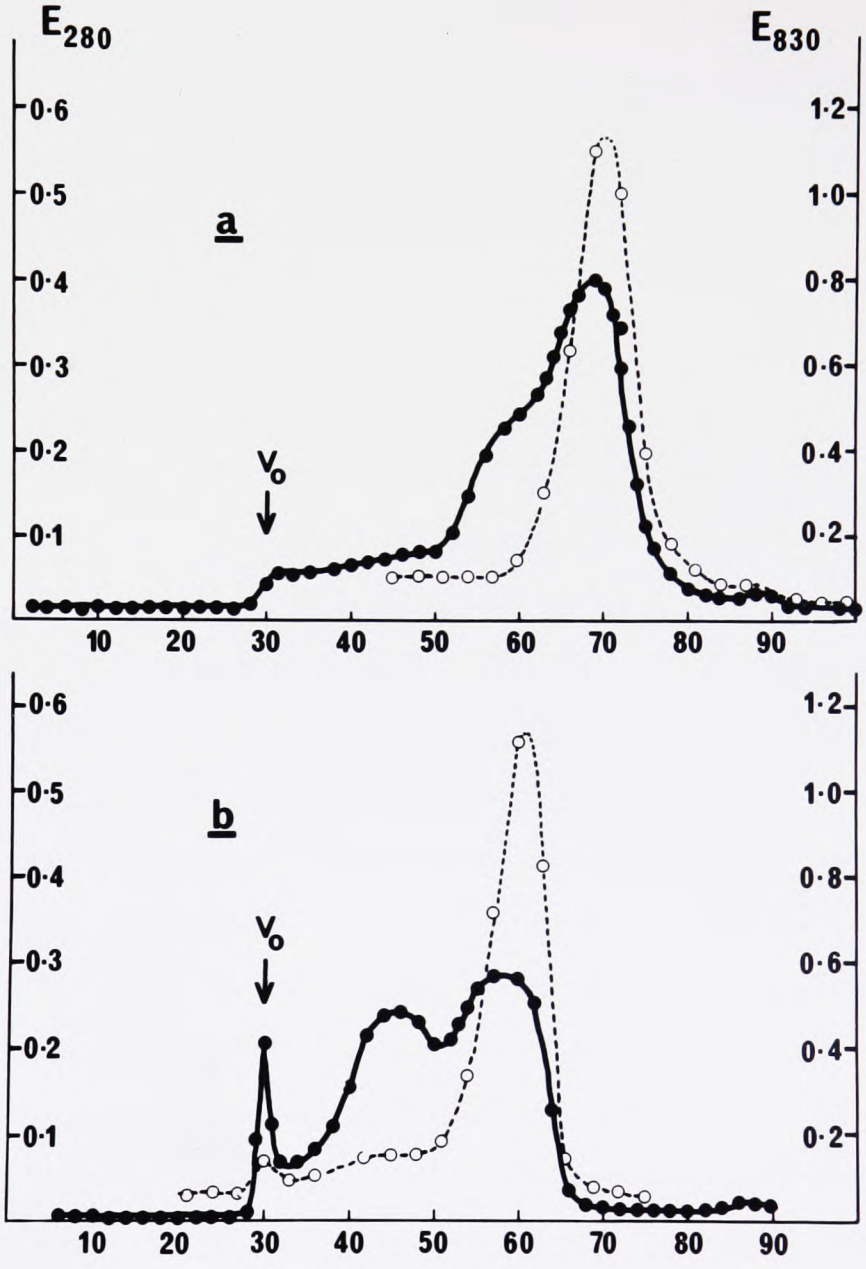
material was eluted after the second major peak, except for some unidentified substance which was eluted as a very small peak in the vicinity of the total volume of the column and therefore is of low molecular weight.

The most important discrepancy between the results of the Innsbruck group and those reported here is the difference in the elution position of the membrane phospholipids. Hörtnagl et al. (1971) found that the phospholipids were completely resolved from chromomembrin B, while I observed no significant separation between these two components (see: Fig. 3.1b). Although Hörtnagl et al. (1971) performed a lipid-extraction with diethyl ether which was omitted in the work described here, it was found that such an extraction did not affect the resolution of the phospholipids from chromomembrin B (see under "Chromatography on Sepharose 6B").

The separation of the peaks observed with the G-200 column was not great and, since only ca. 1/3 of the potential resolving power of the column was utilised under these conditions, it seemed likely that better results might be obtained if fractionation was carried out on one of the agarose gels, which are more suited to the chromatography of high molecular weight substances than Sephadex G-200.

Chromatography on Sepharose 4B.

Since I had already departed from the gel-filtration methodology of Hörtnagl et al. (1971), by substituting agarose gel for Sephadex, and had abandoned the borate/SDS electrophoretic system devised by


E280
E830
a
b
Vo
Vo
0·6
0·5
0·4
0·3
0·2
0·1
1·2
1·0
0·8
0·6
0·4
0·2
10
20
30
40
50
60
70
80
90

Winkler et al. (1970), on the grounds detailed earlier, I decided at the same time to modify the conditions of the gel-filtration system, by lowering the SDS concentration to 0.1%(w/v) - the concentration used in the electrophoretic gels - and by replacing the rather alkaline borate buffer (pH 9.6) with a tris-HCl buffer with a pH (7.8) closer to neutrality.

In a pilot experiment, aimed at establishing the optimum bed material, an aliquot of SDS-dissolved chromaffin granule membranes (in tris-HCl buffer, 1% in SDS) was chromatographed on a small (60cm x $4cm^2$ cross-sectional area) column. The elution profiles of the proteins and phospholipids are shown in Fig. 3.2(a). Once again, the phospholipids were not separated from the trailing peak of protein, but in this case the proteins were not well resolved from one another, indicating that the gel matrix was too "open" for efficient sieving of the proteins (which normally leads to their separation on the basis of molecular radius). The results of this experiment suggested that a bed material with a mean pore diameter intermediate between that of Sepharose 4B and that of Sephadex G-200 would give superior results, and Sepharose 6B was chosen for further experiments.

Chromatography on Sepharose 6B.

The small plastic column employed in the experiment with Sepharose 4B (described above) was used for the first, exploratory gel-filtration in which Sepharose 6B was used as the stationary gel phase. All the experimental conditions were made as close as

possible to those of the previous experiment and Fig. 3.2 (b) shows the elution profiles of chromaffin granule membrane proteins and phospholipids obtained with Sepharose 6B.

Two distinct peaks of ultraviolet-absorbing material were observed, in addition to a small peak at the void volume of the column. The separating capacity of the column was more fully utilised than in the original Sephadex G-200 method, since material was eluted up to a K_{av} of 0.55 from the Sepharose 6B column, whereas no more material was eluted from the Sephadex G-200 column after a K_{av} of 0.39. The material in the void volume of the Sepharose 6B column was of very high molecular weight, since it did not enter a 5% polyacrylamide electrophoresis gel, and it was probably fragments of undissolved membrane which had not been removed by centrifugation. The quantity of this void-volume material varied somewhat in different experiments.

Perhaps the most striking feature of this experiment was that, once again, the phospholipids were not separated from the final protein peak. In an effort to establish whether the variation in methodology from Hörtnagl et al. (1971), who carried out a lipid-extraction before solubilising the membranes, was responsible for this discrepancy in results, the whole experiment was repeated after extracting the membranes, suspended in 1M sodium chloride, twice with aliquots of diethyl ether prior to SDS-solubilisation (the method used by Hörtnagl et al. (1971)). This extraction removed much, but not all, of the lipid content of the membranes. The profiles

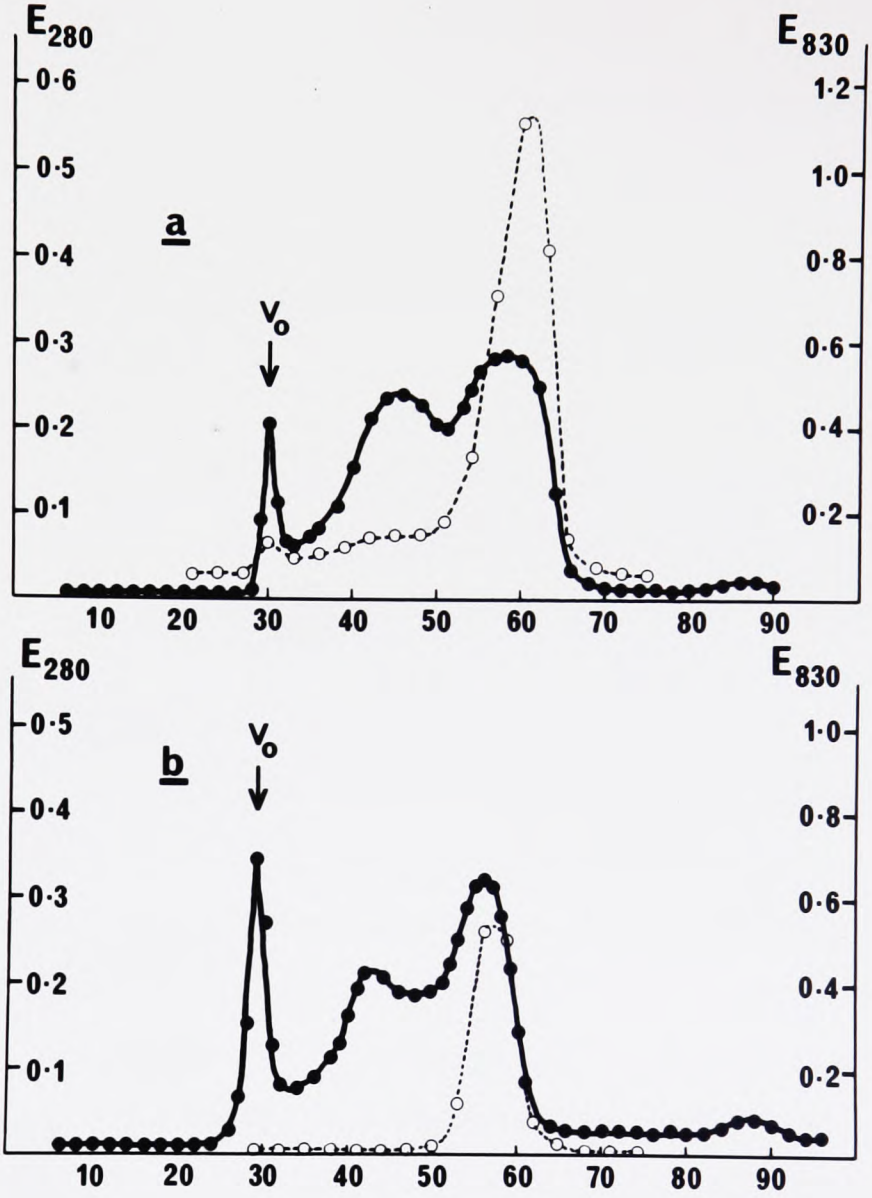


Fig. 3.3. The [illegible] of lipid-containing on Gel-filtration on Sepharose 6B.

a) Unextracted membranes (this is the experiment illustrated previously in Fig. 3.2.b)

b) [illegible] extracted membranes.

The elution profile of the phospholipids is shown by a broken line in both figures and the units of the abscissa are fraction numbers in both figures.

obtained with this (extracted) sample on gel-filtration are shown in Fig. 3.3 (b), in which they are compared with the profiles obtained in the previous experiment with unextracted membranes. The elution position of the remaining phospholipids was unaffected by this treatment and the difference between these results and those reported by Hörtnagl et al. (1971) remains, therefore, unexplained. The void-volume peak is rather larger in this experiment than in the previous one since the SDS-extract was not centrifuged before being applied to the column.

Preparative-Scale Chromatography on Sepharose 6B.

Since these preliminary, small-scale experiments with Sepharose 6B as bed material indicated that resolution of the proteins comparable to, or even perhaps better than, that reported by Hörtnagl et al. (1971) was to be expected from this alternative method, a larger column (90cm x 8cm^2 cross-sectional area) was packed with Sepharose 6B which had been equilibrated with the tris-HCl/SDS buffer, and this "large column" was used for attempts to purify the membrane proteins in milligramme quantities.

It was evident from the first such preparative experiment that this large column afforded a considerably improved separation (see elution profiles in Fig. 3.4) of the constituent membrane proteins, when compared with the published profile of Hörtnagl et al. (1971). With the increased resolution of the larger column, each of the two peaks observed in the pilot (small column) experiments were seen to

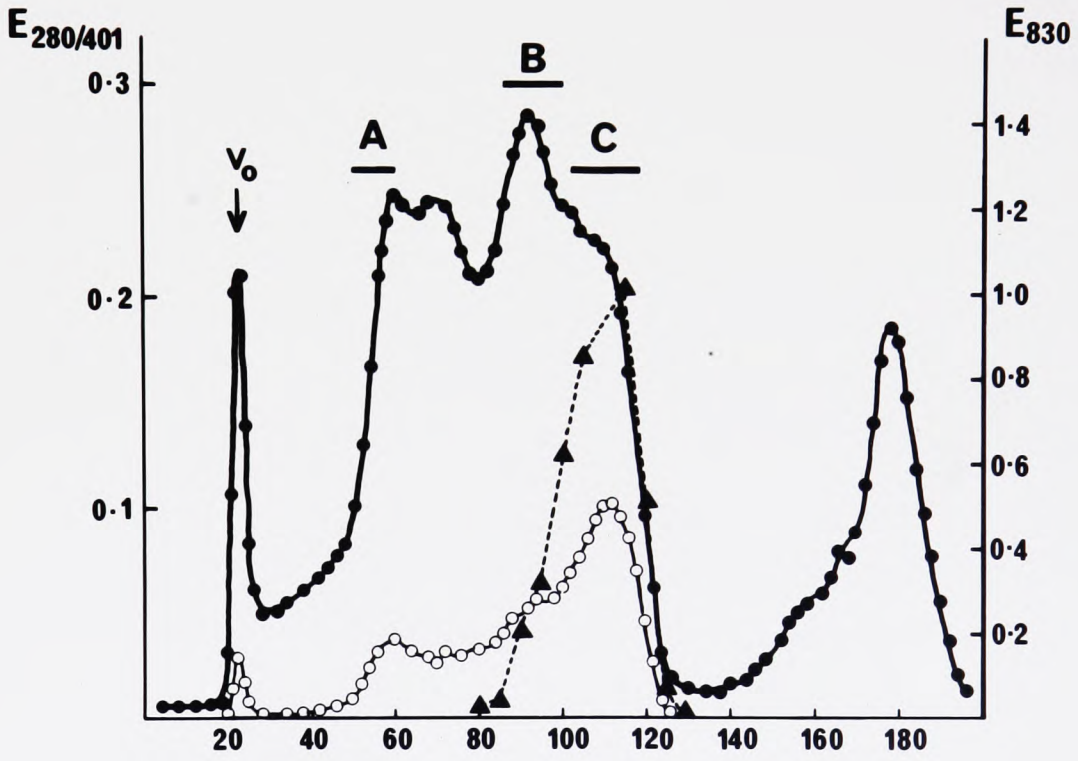


Fig. 3. Negative Col-filtration on Agarose Sepharose 6B Column.

Optical density at 280nm: ●—●—●

Optical density at 401nm: ○—○—○

Phospholipid reaction (measured extinction at 830nm): --▲---▲---▲--

The units of the abscissa in this and all succeeding figures are fraction numbers. The fractions under the bars were pooled to provide samples for rechromatography.

consist of more than one component. The first major (non-excluded) peak was resolved into two overlapping components, while the second peak was found to have two "shoulders" on the descending side.

Electrophoretic analyses of selected column fractions showed that the fractions on the rising side of the first peak contained chromomembrin A as the major component, free of the high-molecular weight aggregated material, but increasingly contaminated by faster-running components towards the peak maximum. (see Plate 3.1). This analysis also revealed that the major component of the fractions around the maximum of the second major peak (the largest peak in the profile of absorbance at 280nm versus fraction number, in Fig. 3.4) was chromomembrin B, and that this protein was also contaminated by minor components (slower-running in this case). In agreement with the report of Hörtnagl et al. (1971), it was found that component C, eluted between chromomembrin A and chromomembrin B, was always markedly contaminated by other proteins and could not be obtained in a sufficiently homogeneous state for amino acid analysis to yield a meaningful result.

After measuring the absorbances of the fractions at 280nm, which detects (non-specifically) proteins and certain non-protein substances such as nucleic acids, nucleotides, catecholamines etc., some fractions were re-examined to determine their absorbance at 401nm, a wavelength near the absorption maximum of the membrane cytochrome under these conditions. It is noteworthy that absorbance at this wavelength was not confined to a single peak, but was distributed

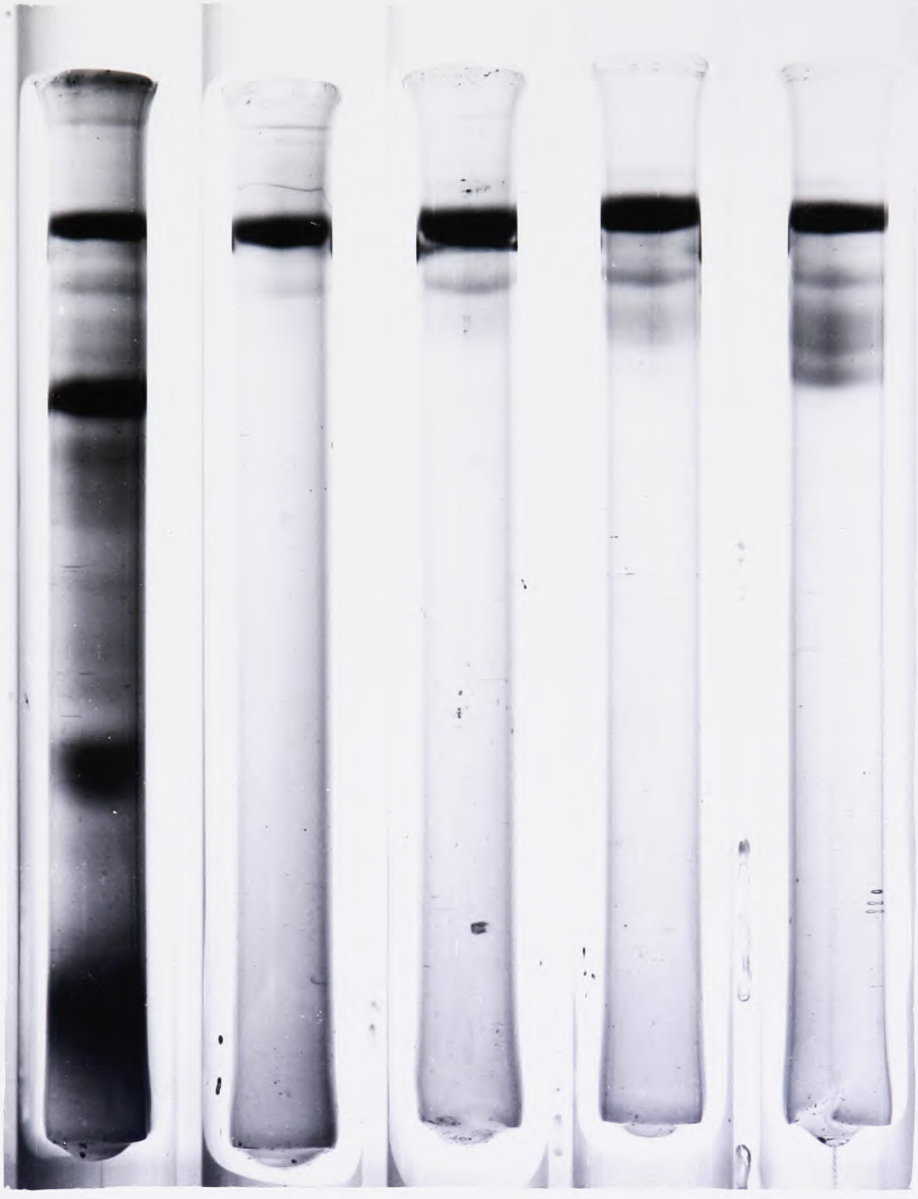


Plate 3.1. Electrophoretic Patterns of Selected Fractions of the Effluent from the Preparative Gel-filtration.

Aliquots of the effluent from individual fractions from the preparative gel-filtration shown in Fig. 3.4 were analysed by electrophoresis in 6% polyacrylamide gels. Reading from left to right, the fractions were: total membranes (for comparison), fractions 52, 56, 60 and 62.

throughout nearly all of the fractions containing eluted proteins. The elution profile at 401nm did not mirror that at 280nm in its overall shape and, for convenience in description, it may be divided into two sections: firstly, a reasonably symmetrical peak, spanning fractions 100 to 120, and, secondly, a region of lower absorption showing a distinct peak around fraction 60, followed by an irregular rise to the leading edge of the major peak. (The small peak in the void-volume probably indicates, once again, the presence of small amounts of unsedimented membrane fragments). When the ratio of absorbance at 401nm to absorbance at 280nm was calculated, it was found to resemble the profile of absorbance at 401nm in that there was a distinct peak near fraction 58, (E_{401}/E_{280} = 0.15) followed by a rather flat plateau rising to a sharp peak with a maximum in the region of fraction 112 (E_{401}/E_{280} = 0.52). The E_{401}/E_{280} ratio in this peak fraction (0.52) is almost exactly double that recorded for the unfractionated sample of SDS-dissolved membranes (0.26 - 0.27), however the interpretation of this observation is complicated.

In this experiment, the absorbance at 401nm was not measured in fractions after number 126 (the following fractions appearing uncoloured, unlike the fractions making up the major peak, which were perceptibly orange) and thus no estimate can be made of the total recovery of cytochrome, based on "401nm absorbance".

However, the eluate was monitored at 280nm up to a position after the (calculated) total volume of the column and, knowing the

absorbance of the original sample (measured spectrophotometrically, using aliquots of the original sample diluted with column buffer to give an absorbance in the region of that observed in the eluted fractions), it is possible to estimate the recovery of "280nm absorbance" by measuring the area under the elution profile, making the assumption that the extinction coefficients of the constituent proteins were not affected by the chromatographic separation. When the recovery of "280nm absorbance" was calculated in this fashion, it was found to be 98.1% of the material applied (i.e.: within experimental error, the recovery from the column was quantitative).

When a similar calculation was made for the recovery of "401nm absorbance", it was found that only 60.0% of the applied material was recovered in those fractions whose absorbance at 401nm had been measured. Thus, if the cytochrome was also recovered quantitatively, 40% must have been eluted in the fractions after number 126, which were not measured.

Approximately 13.1% of the recovered "280nm absorbance" was eluted in fractions 100 to 119 inclusive and it is interesting, in view of the observed E_{401}/E_{280} ratios noted earlier, that, taking into account the "missing" 40%, 26.45% of the "total" 401nm absorbance was also located in these fractions. In other words, it may be that these fractions contain the cytochrome enriched only twice with respect to the original starting material. Since the other proteins appear to be rather more effectively purified on gel-filtration, it

is possible that some property of the cytochrome itself is responsible for this poor purification.

The Nature of the Material in the Final Peak.

Although most interest naturally centres around the proteins eluted from the column, the final peak, which was also noted in experiments with bed materials other than Sepharose 6B and which has been assumed to represent low molecular weight substances, was sufficiently large to make a substantial contribution to calculations of recovery based on "280nm absorbance" and attempts were therefore made to try to discover its chemical composition. The peak normally took the form of a symmetrical curve with a conspicuous "shoulder" on the ascending limb, but none of the methods employed showed any distinction between the properties of these two chromatographic components.

The ultraviolet absorption spectrum of the material eluted in this final peak showed a distinct absorption maximum in the region 280 - 285nm, but no peak was observed in the 225 - 250nm region where proteins have a strong absorption due to peptide bonds. It was found that this material did not react detectably with Folin & Ciocalteau's reagent and, since the amount of material available was very small, spectrofluorimetry was the only technique with sufficient sensitivity to permit further analysis.

The emission spectrum of the unknown material consisted of a single peak, centred on 370nm. The position of this (emission)



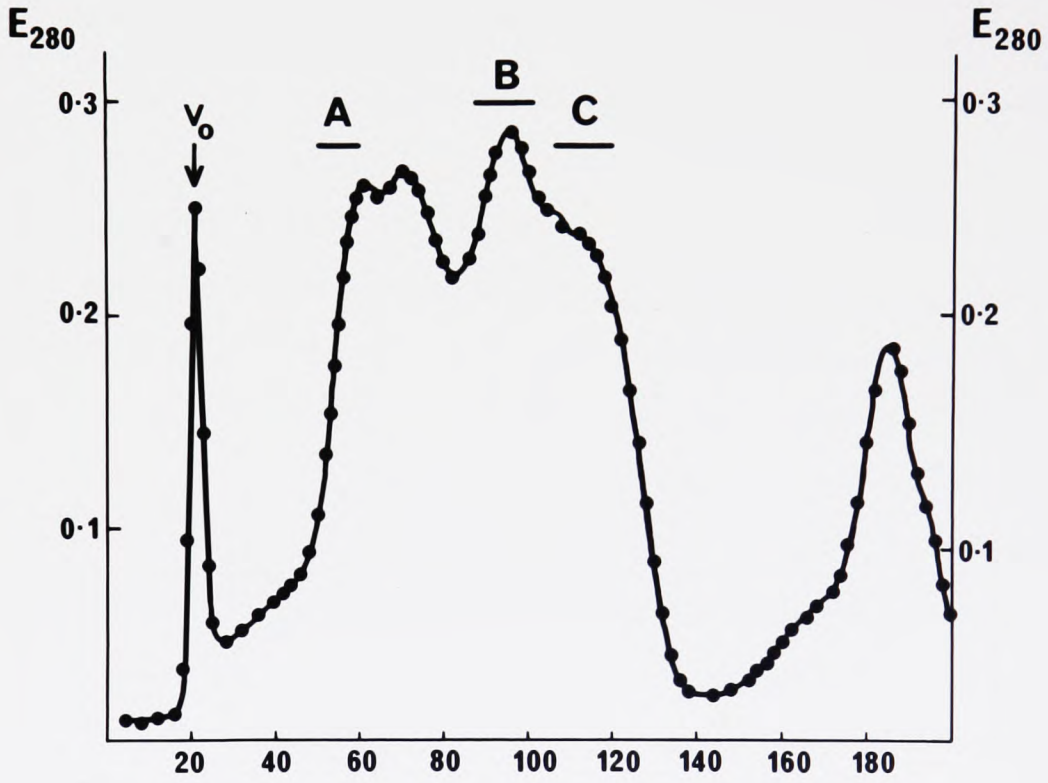
peak was independent of the wavelength of excitation, over the range 248 - 335nm (see Fig. 3.5), although the height of the peak (which is a measure of the quantum efficiency of the fluorescence process) was dependent on the wavelength at which the fluorescence was excited, with maxima at (excitation) wavelengths of 270 - 275, 300 and 335nm.

The excitation spectrum consisted of three overlapping peaks when the emission was monitored at 370nm and the maxima of these peaks lay at wavelengths of 270, 300 and 335nm. The shape of the excitation spectrum was dependent on the wavelength at which the fluorescence emission was observed, partly through light-scattering effects, but the wavelengths at which the excitation maxima occurred did not appear to be affected.

By contrast, the eluate from the column in those regions marked "A" and "B" in Fig. 3.4 and Fig. 3.6 had a single excitation maximum, at a wavelength of 290nm - characteristic of tryptophan residues in proteins - and its emission spectra showed fluorescence maxima at 348 - 350nm (major peak) and 628 - 633nm (minor peak). Under the same conditions bovine serum albumin was found to have a single excitation maximum, at a wavelength of 288nm, and two emission maxima, at wavelengths of 325nm (major peak) and 612nm (minor peak).

Further Purification of Individual Proteins by Rechromatography.

Examination of the electrophoretic patterns given by individual column fractions showed that a single column chromatography step was not sufficient to give an homogeneous preparation of any of the

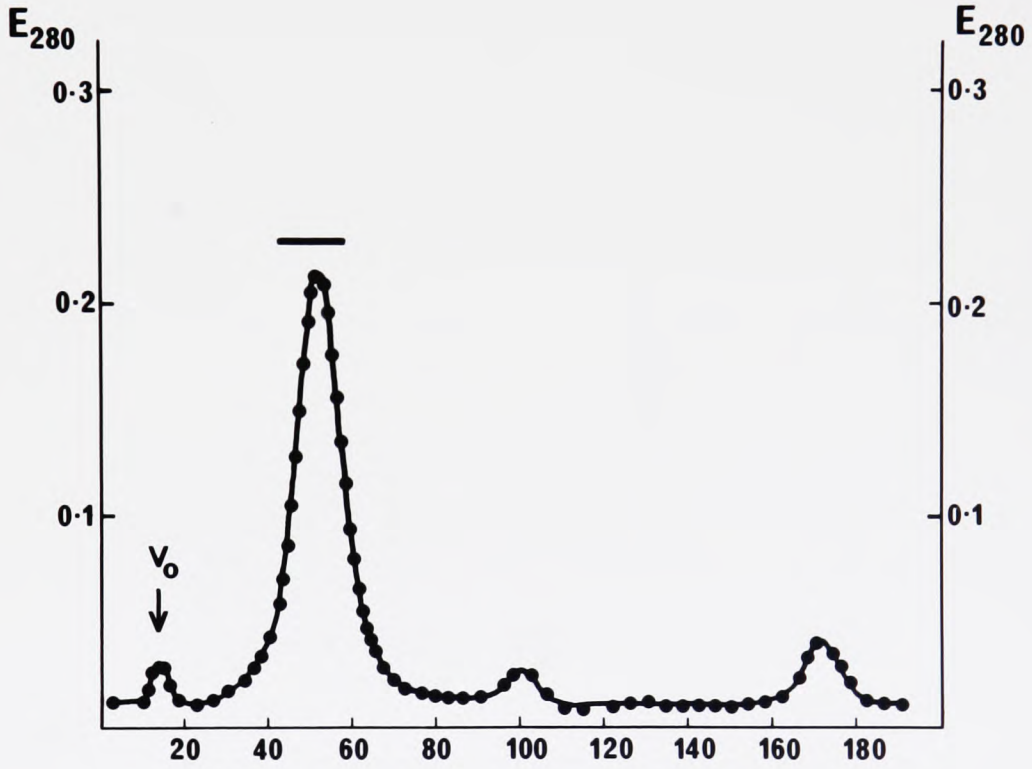

E280
E280
0·3
0·2
0·1
Vo
A
B
C
20
40
60
80
100
120
140
160
180

major chromaffin granule membrane proteins. However, the separation between the various proteins on this large Sepharose 6B column was sufficiently good to engender the hope that all but the last traces of the contaminants might be removed if the major proteins were submitted to rechromatography on the same column. In order to have sufficient starting-material to make such rechromatography worthwhile, another batch of chromaffin granule membranes was prepared, solubilised with SDS and chromatographed on the large Sepharose 6B column, exactly as before. The elution profile from this second experiment, which was monitored at 280nm only, is shown in Fig. 3.6 and was found to be virtually identical with that obtained in the first experiment (cf: Fig. 3.4). The recovery of "280nm absorbance" in this second separation was 99% of the applied material.

Fractions from three regions of the elution profile (marked "A", "B" and "C" and comprising those fractions included within the black bars in Fig. 3.4 and 3.6) were pooled and the corresponding solutions from each experiment were combined and ultrafiltered at room temperature, to give a final volume of ca. 6ml. These ultrafiltered samples were then used for rechromatography.

Rechromatography of Fraction A.

"Fraction A" was composed of the material eluted on the rising side of the first (non-excluded) peak in the elution profile given by SDS-dissolved membranes, and it consisted of chromomembrin A contaminated with small amounts of proteins with higher electrophoretic


E280
0·3
0·2
0·1
Vo
20
40
60
80
100
120
140
160
180
E280
0·3
0·2
0·1

mobilities. On rechromatography of this preparation, four peaks could be distinguished in the elution profile (monitored at 280nm only, see: Fig.3.7). After a very small initial peak, at the void volume of the column, there emerged a smooth, symmetrical, major peak with K_{av} = 0.213, which accounted for 84.5% of the recovered "280nm absorbance". This peak was followed by a small peak with K_{av} = 0.483 and a slightly larger peak with K_{av} = 0.881.

When individual fractions spanning the major peak were analysed electrophoretically, it was found that although the major part of the protein present was chromomembrin A, there was still appreciable contamination (see: Plate 3.2 and Fig. 3.7) by a minor component with a R_m x 100 of ca. 27 (on 6% polyacrylamide gels) and small amounts of other faster-running components. In spite of the presence of these contaminants, the fractions around the major peak were pooled and ultrafiltered at room temperature, for subsequent use. This final preparation had a E_{280}/E_{260} ratio of 1.254 and will be termed "purified chromomembrin A".

Rechromatography of "Fraction B".

"Fraction B" was obtained by pooling the fractions spanning the maximum of the second major peak in the elution profile resulting from the separation of SDS-solubilised membranes. It consisted mainly of chromomembrin B, contaminated by small amounts of proteins with lower electrophoretic mobilities and by the membrane cytochrome, but, in addition, this sample contained appreciable amounts of the

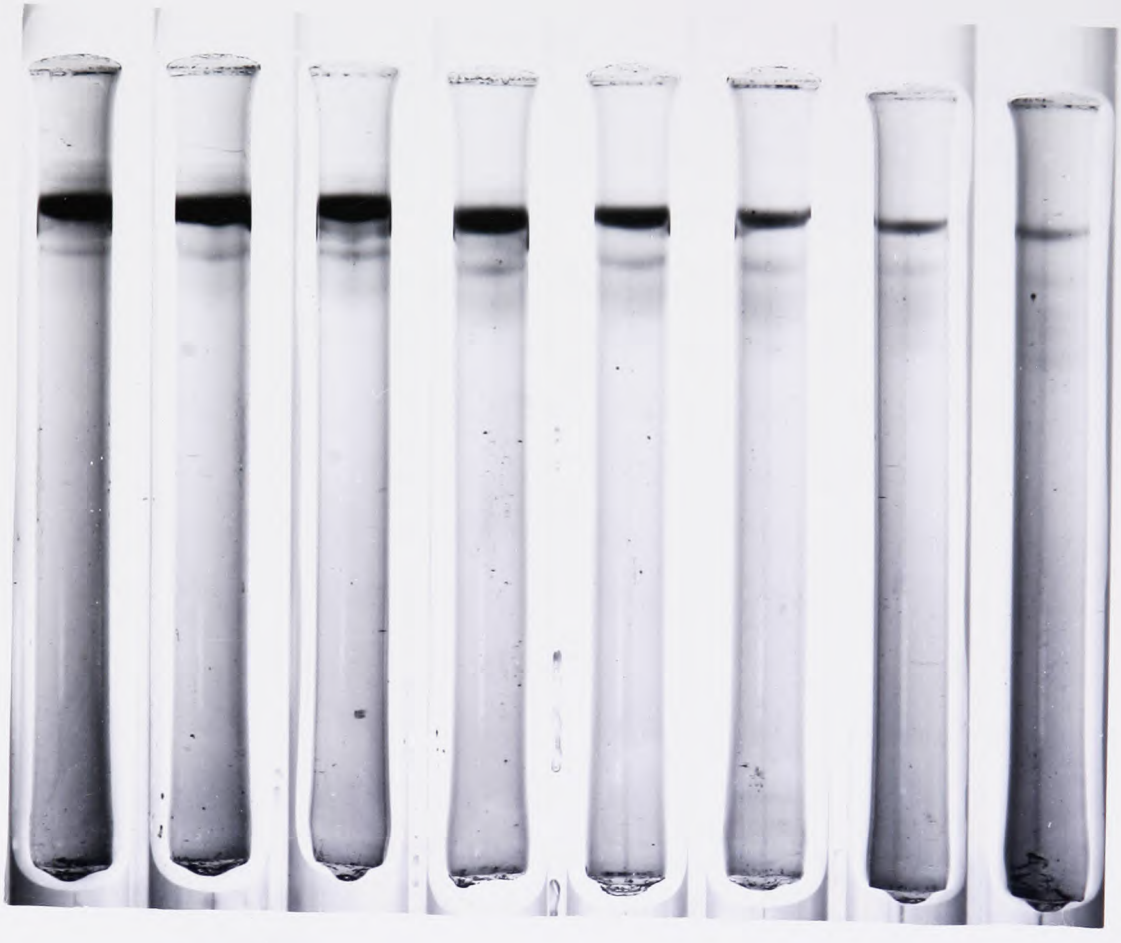


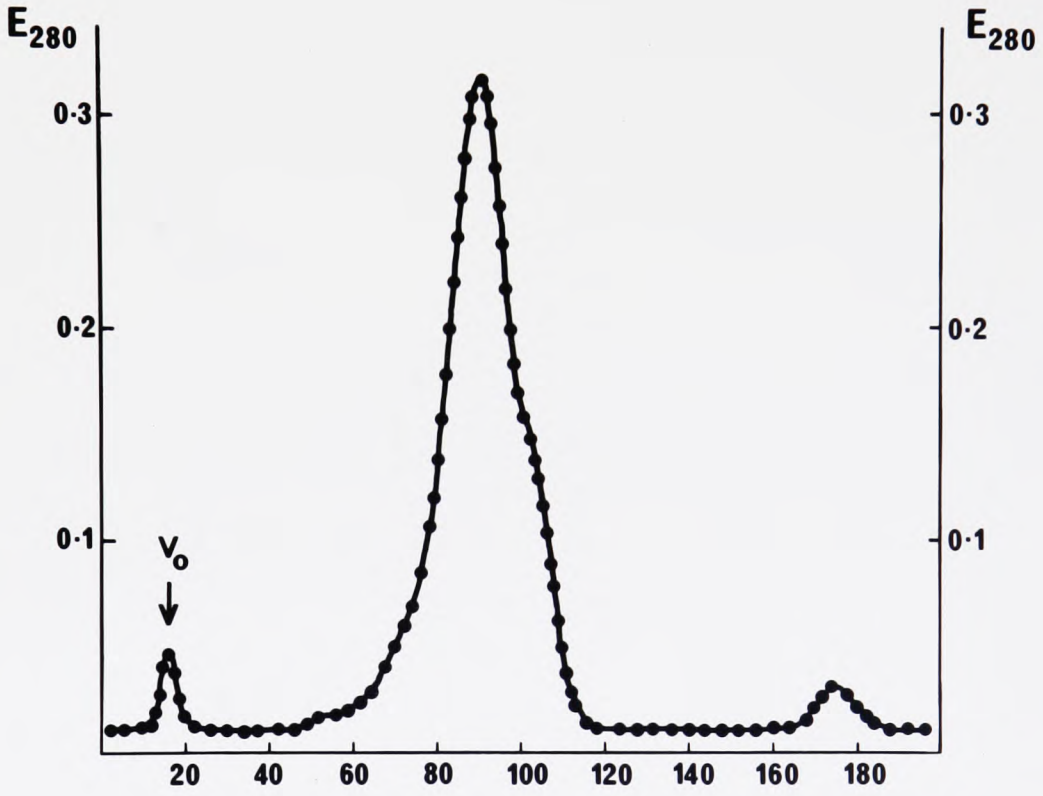
Plate 3.4 Electrophoretic Analysis of the Material Eluted in [illegible] from the Rechromatography of "Fraction A".

As in Plate 3.1., the electrophoresis was carried out in 6% gels. The column fractions analysed were (from left to right): [illegible] 50, 52, 54, 56, 58, 60 and 62. (see: Fig. 3.7.).

membrane lipids.

On rechromatography of "Fraction B", three peaks could be identified in the elution profile of absorbance at 280nm (see: Fig. 3.8). As in the rechromatography of "Fraction A", there were small peaks at the void volume of the column and at K_{av} = 0.880, accounting for 2.46% and 3.09% of the recovered "280nm absorbance" respectively. The remaining 94.45% of the recovered "280nm absorbance" was located in the central, major peak. In this experiment the total recovery of material, calculated from its 280nm absorbance, was 93.1% of that applied.

On close examination of the elution profile, it would appear that the major peak, which had a pronounced "shoulder" on the descending side, was composed of several overlapping components. A very small peak (fractions 50 to 57) preceded the initial rise of the leading edge of the main peak and the absorbance then rose fairly slowly until fraction 75 was reached. Although the slow rise in absorbance may simply be a consequence of peak-broadening (which would be expected to be more pronounced than for chromomembrin A, owing to the larger K_{av} of chromomembrin B) it is conceivable, in view of the detection of slower-running contaminants by electrophoresis, that the envelope of the curve in this region of the elution profile may result from the summation of the absorbances of a number of components, eluted one after another in increasing quantities and preceding chromomembrin B. The upper portion of the


E280
0·3
0·2
0·1
Vo
20
40
60
80
100
120
140
160
180
E280
0·3
0·2
0·1

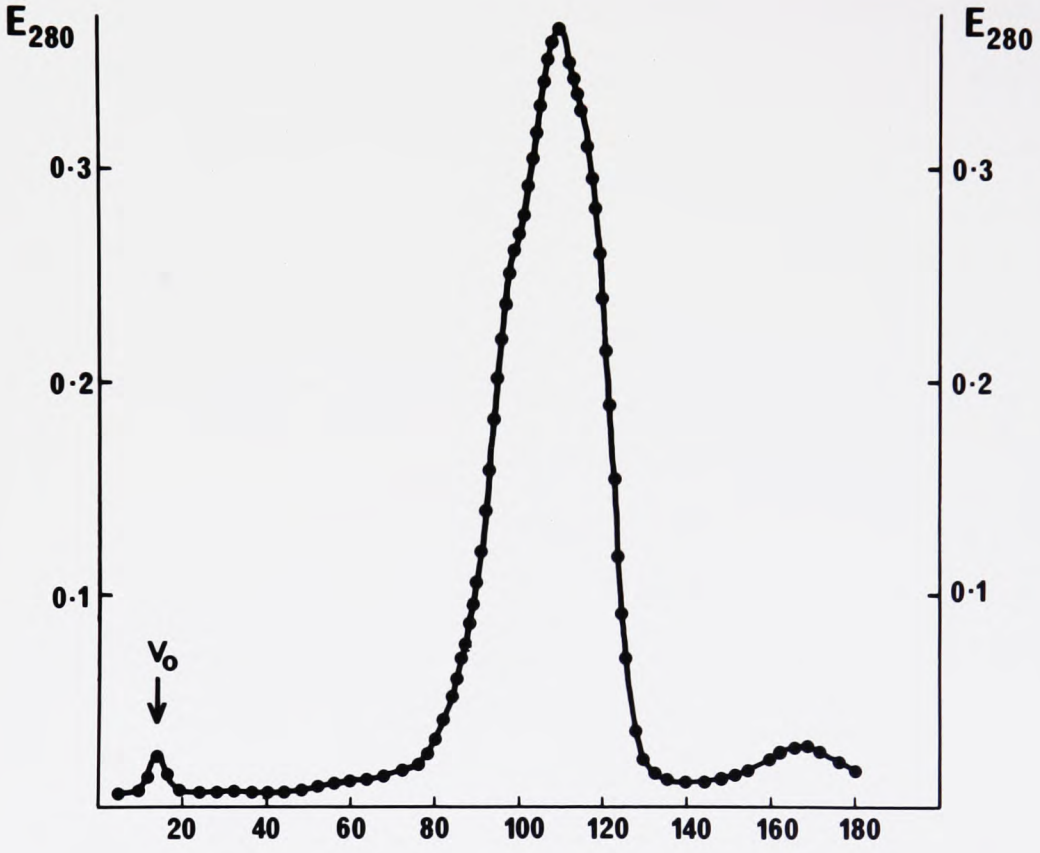
peak (fractions 80 to 95) appeared to be smooth and symmetrical and the marked "shoulder" on the falling limb of the curve was centred around fractions 103 - 104.

Aliquots of fractions 83, 85, 87, 89, 91, 93, 95 and 97 from this rechromatography were further analysed by electrophoresis on 8% polyacrylamide gels. Although the major stained band present in each gel was chromomembrin B (which gave a rather broad band with an R_m x 100 of approximately 64 under these conditions), in no case was this the only observable band. The earlier fractions (83 - 87) contained various amounts of slow-running proteins, at least two of them (with R_m x 100 of 50 and 58) staining quite strongly, whilst the later fractions (91 - 95) gave the diffuse, greenish stained band characteristic for the membrane lipids. The least contaminated fractions were those close to the maximum of the peak (91 and 93).

Rechromatography of "Fraction C".

"Fraction C" consisted of pooled fractions from the "shoulder" region of the descending limb of the second major peak in the elution profile given by SDS-dissolved chromaffin granule membranes. This fraction contained material with 401nm absorption (characteristic of the membrane cytochrome), some chromomembrin B and much of the eluted membrane lipids.

When "Fraction C" was ultrafiltered and submitted to rechromatogrsphy, three peaks were evident in the elution profile (Fig. 3.9), which was monitored at 280nm only. As in the case of


E280
0·3
0·2
0·1
Vo
20
40
60
80
100
120
140
160
180
E280
0·3
0·2
0·1

the rechromatography of "Fraction B", the three peaks observed consisted of a minor peak at the void-volume of the column, a minor peak near the (calculated) total volume and a major, central peak, which appeared to have more than one chromatographically distinguishable component.

When "Fraction C" was ultrafiltered to concentrate it to a small volume suitable for application to the column for rechromatography, the diffusible material which passed through the dialysis tubing (and was, therefore, not concentrated) did not appear completely colourless. Unfortunately, this material was not analysed or retained. After rechromatography, the original orange-brown colour of the concentrated "Fraction C" sample was so attenuated by dilution that no peak could be discerned by eye, and even spectrophotometric analysis failed to detect a significant peak of "401nm absorbance".

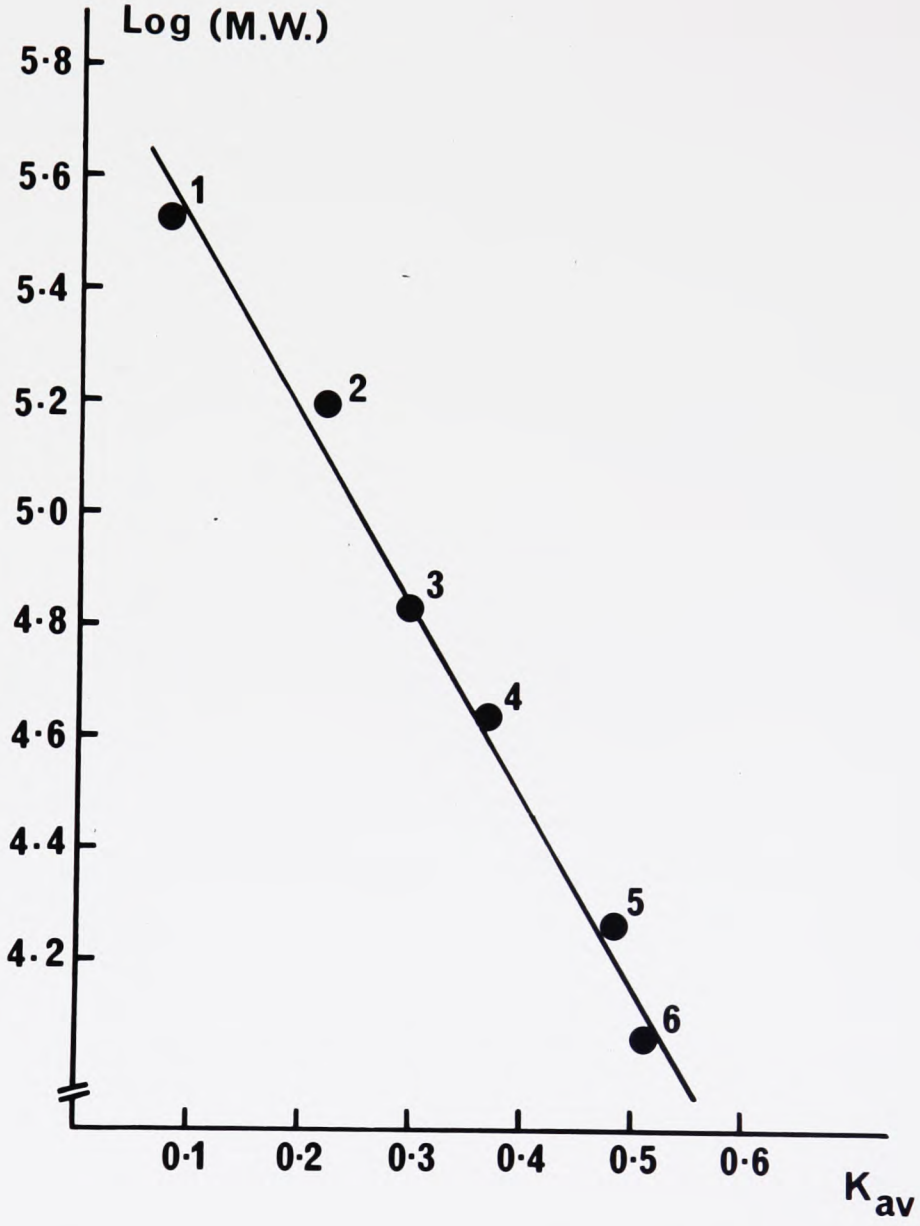
Electrophoretic analysis, once again on 8% polyacrylamide gels, of column fractions spanning the major peak in Fig. 3.9 revealed small amounts of chromomembrin B to be present in the earlier fractions (97 and 100), but only the green lipid band could be seen in later fractions (106 - 118). While it is possible that some cytochrome protein might have been present in the fractions but have escaped detection because it was not fixed or stained, or was not distinguishable from the broad band of lipid, the very low colouration of the samples may suggest that treatment with SDS during the solution of the membranes, the subsequent chromatographic separation

and analysis, and the concentration of "Fraction C" by ultrafiltration, followed by rechromatography, may have resulted in dissociation of the cytochrome, with attendant loss of its characteristic visible absorption spectrum.

Estimation of Molecular Weights by Chromatography.

Since proteins are separated on the basis of their effective hydrodynamic radii during gel-filtration (Ackers, 1964), and since these radii are in turn linearly related to molecular weight for the majority of globular proteins, molecular weights may be estimated from gel-filtration measurements if the column is "calibrated" with standard proteins. A large number of papers have appeared, describing such methods for determining molecular weights of undenatured proteins in dilute buffers (Ackers, 1964; Andrews, 1964), or of denatured proteins in solutions containing guanidine hydrochloride (Fish, Mann & Tanford, 1969), sodium dodecyl sulphate (Fish, Reynolds & Tanford, 1970), phenol/acetic acid (Pusztai & Watt, 1970), or other denaturing agents.

The large Sepharose 6B column, used for the preparative separations and rechromatography experiments just described, was "calibrated" by measuring the elution volumes of six standard proteins (thyroglobulin, γ-globulin, bovine serum albumin, ovalbumin, β-lactoglobulin and cytochrome c) and constructing a graph of the logarithm of molecular weight versus K_{av}. The quantity K_{av} was preferred to K_d when constructing this graph because it is slightly


Log (M.W.)
5·8
5·6
5·4
5·2
5·0
4·8
4·6
4·4
4·2
1
2
3
4
5
6
0·1
0·2
0·3
0·4
0·5
0·6
K_{av}

less sensitive to errors in the determination of the elution volumes and because it does not make use of the "inner volume" V_i - a quantity which is not as accurately measurable as V_t (the total volume of the column).

Only one measurement was made of the elution volume of each of the standard proteins and the scatter of the points around the computed regression line for the "calibration graph" (see: Fig. 3.10) is rather greater than that observed in the electrophoretic molecular weight calibration graphs. The greater scatter is partly a consequence of the smaller number of experimental points used to construct the calibration graph for gel-filtration, but some of the scatter may be attributed to the facts that the proteins are much less well resolved from one another than is the case in electrophoretic separations and that the elution volumes cannot be measured with the same precision as relative mobilities. The correlation coefficient for the gel-filtration calibration graph was -0.9926.

When constructing the graph, the molecular weight of thyroglobulin was taken to be 335,000 (see: Reithel, F.J., 1963; also Edelhoch & Hall, 1964). A second (minor) peak was, however, also observed in the elution profile and this probably represents the "monomer" form, which has a molecular weight of 165,000 (Charlwood, Pitt-Rivers & Schwartz, 1970). This component had a K_{av} which lay close to the expected value for the "monomer" on the calibration graph, but it was not included when computing the regression line.

Table 3.1

K_{av} and Molecular Weights of Standard and SDS-dissolved Chromaffin Granule Membrane Proteins from Chromatography on Sepharose 6B.

Standard Proteins.	Mol. Wt.	Measured K_{av}.
Thyroglobulin	335,000	0.079
γ-Globulin	157,000	0.222
Bovine Serum Albumin	68,000	0.296
Ovalbumin	43,000	0.368
β-Lactoglobulin	18,400	0.490
Cytochrome c	11,700	0.507

Chromaffin Granule Proteins.	Measured K_{av}.	Estimated Mol. Wt.
Chromomembrin A (first prep.)	0.213	135,900
" (second prep.)	0.214	135,150
" (rechromatog.)	0.213	135,990
Chromomembrin B (first prep.)	0.393	34,100
" (second prep.)	0.401	31,990
" (rechromatog.)	0.415	28,780
Cytochrome (first prep.)	0.499	15,070
"Component C" (first prep.)	0.269	88,270
" (second prep.)	0.262	93,300

Two calibration experiments were performed. In each, the sample applied to the column contained three of the standard proteins (chosen so that they were well resolved from one another) and a quantity of D-glucose. The elution volume of the glucose was determined from colorimetric analysis of the column fractions with anthrone reagent, and was found to be 669ml in the first experiment and 678ml in the second experiment. The maximum of the final peak observed on separation of SDS-dissolved chromaffin granule membranes also occurred in the region of these elution volumes and they could be taken to be estimates of the "inner volume" of the column. However, as has already been noted, this quantity was not used when constructing the calibration graph and the above observations were therefore merely taken as evidence to indicate that the unknown material in the final peak was of low molecular weight.

The K_{av} values measured for the standard proteins by this column procedure are set out in Table 3.1, along with the K_{av} values of some of the chromaffin granule membrane proteins and estimated molecular weights derived from these figures using the calibration graph shown in Fig. 3.10.

CHAPTER 4.

Discussion of the Electrophoretic and Gel-filtration Experiments.

Page

Discussion

The electrophoretic system. 79

The molecular weight estimates 81

Quantitative densitometry 87

The gel-filtration experiments 90

Alternative purification methods 92

Prospect 94

DISCUSSION

The initial stimulus which led me to undertake the studies described in the preceding two chapters was the description by Hörtnagl et al.,(1971) of the borate/SDS gel-filtration method, which seemed to offer the possibility of obtaining pure preparations of at least the two major chromaffin granule membrane proteins in sufficient quantities for chemical studies of their structures and compositions to be practicable. The Innsbruck group had described, in an earlier paper (Winkler et al., 1970), the preparation of the chromaffin granule membranes and had characterised this preparation in terms of the enzymes present and the contents of various non-enzymic substances, such as lipids and chromogranin A, and they had described electrophoretic systems in which the membrane proteins could be separated analytically. They used the more convenient of these (the borate/SDS system) to demonstrate the effectiveness of their gel-filtration purification method (Hörtnagl et al., 1971), but they did not attempt to measure the molecular weights of these various proteins.

The Electrophoretic System.

Relatively brief practical experience of their electrophoretic method sufficed to convince me that this borate/SDS system is not, in fact, a suitable system for either routine analysis of chromaffin granule membrane proteins or for the estimation of their molecular weights. The mechanical weakness of the 3.5% gels makes them very

prone to damage during and after removal from the running-tubes, and they show an alarming degree of shrinkage when immersed in the methanolic stain/fixative (of DeVito & Santomé, 1966) recommended by Winkler et al., (1970). Furthermore, the relatively high concentration of SDS in the gels (1%) is a potential disadvantage, since it may be expected to make fixation of the protein bands more difficult. The use of 6% gels circumvents some of the mechanical problems, since these more concentrated gels are much stronger and less liable to shrink. The fixation was made more sure by reducing the SDS concentration in the gels and electrolyte to 0.1% and by substituting 25% trichloroacetic acid for the 50% methanol/10% acetic acid stain/fixative of DeVito & Santomé. Raising the gel concentration also improved the sharpness of the bands, although increasing the time needed for electrophoresis, and all of the proteins seen in 3.5% gels still entered the 6% gels. Indeed, the gel concentration could have been raised to at least 9% without causing any of the proteins to be excluded (see: Plate 2.1) and 8% gels were used to analyse mixtures, such as column fractions, containing only the faster-running components. An estimate of the limit at a particular gel concentration above which molecular weight no protein will enter the gel, may be obtained from the log (molecular weight) versus $R_m \times 100$ "calibration graph", by extrapolation of the regression line to $R_m \times 100 = 0$. When this was done with the data used for the present studies, the exclusion limit was found to lie at a molecular weight of 355,000 for a 5% gel and at 183,000 for a 6% gel (both figures

relating to reduced proteins - with unreduced proteins, the limit for 6% gels was 219,000).

The Molecular Weight Estimates.

With this improved electrophoretic system, it was possible to embark on molecular weight studies. All of the literature methods for measuring molecular weights by electrophoresis direct that disulphide-bonds within or between polypeptide chains should be broken, by exposing the proteins to reducing reagents such as β-mercaptoethanol or dithiothreitol, and early experiments of this kind (on 5% gels) served to indicate probable molecular weights for the majority of the components of the chromaffin granule membrane protein pattern. However, there was a particularly unfortunate overlap between chromogranin A (which, contrary to the report of Kirshner & Kirshner (1969), was not split into smaller fragments on reduction) and the sub-unit of dopamine β-hydroxylase, and this overlap was not appreciably lessened by raising the gel concentration to 6%. Since a satisfactory separation of all of the bands was achieved when reducing-agent was omitted from the sample, I decided to try to determine molecular weights by electrophoresis of unreduced proteins.

The observation that the electrophoretic behaviour of various chromaffin granule membrane proteins deviates from the linearity predicted by the Ferguson equation (see: Fig. 2.2) is, perhaps, not altogether unexpected, since several reports (Bretscher, 1971;

Segrest et al., 1971; Evans & Gurd, 1973) of membrane proteins showing this kind of effect have appeared. Indeed, it is likely that this phenomenon is more general than is currently recognised, and normally escapes attention because most investigators do not trouble to determine "molecular weights" at more than one acrylamide concentration. It is certainly preferable that such studies should be performed before electrophoretically-determined molecular weights are assigned to relatively uncharacterised proteins, particularly if they are membrane proteins.

Hörtnagl et al., (1971) found that chromomembrin B ran very slowly (more slowly than chromomembrin A) in gels containing phenol/acetic acid/urea (under which conditions most proteins once again migrate according to the order of their molecular radii) and, since they found that it moved very rapidly in gels containing the borate/SDS buffer system, they explained these observations by suggesting that this protein might have a very compact conformation in the alkaline (borate/SDS) system, but a very extended conformation in the acid system. While my studies did not confirm or disprove this hypothesis, chromomembrin B was found to have absolutely regular electrophoretic properties in the phosphate/SDS system used for my work, and it is likely that the values of 28,485 and 28,728 (Table 2.3) recorded for its molecular weight, in the presence and absence of reducing-agent respectively, are good estimates of the true figure.

What can be concluded about the molecular weights of the other

chromaffin granule membrane proteins (since most of them show non-linear electrophoretic behaviour)? The estimated molecular weights of chromogranin A (component C) are 79,076 (reduced, in 5% gels), 77,377 (reduced, in 6% gels) and 80,319 (unreduced, in 6% gels) and, despite the fact that this protein deviated from linearity in the Ferguson plot (Fig. 2.2) and was found to give the positive reaction expected for a glycoprotein with the periodic acid/Schiff reagent stain (Fig. 2.7), these estimates are close to the molecular weight of 77,000 reported by Smith & Winkler (1967b), from amino acid analysis and ultracentrifugal sedimentation data obtained with the purified protein. The observed difference between the value recorded with reduced and unreduced samples on 6% gels may mean that the unreduced proteins give molecular weights which are slightly too high, and it could be that this indicates that the denatured proteins still retain a small amount of their native structure, not completely destroyed by SDS, which could cause a lowering of their electrophoretic mobilities (and hence higher apparent molecular weights) when compared with the (presumably completely denatured) reduced forms.

Other components which showed linear Ferguson plots, and therefore probably gave reliable molecular weight estimates, were all minor components of the staining pattern. Thus, the molecular weight of 37,895 calculated for the minor component shown in Fig. 2.2 is probably a valid estimate and the molecular weight of component "F"

(see: Fig. 2.7 - this component was not plotted in Fig. 2.2, for the sake of clarity, but also gave a straight line) is likely to lie close to the estimates of 69,291 and 69,558 recorded on reduced and unreduced gels respectively.

It is, however, rather less easy to interpret the molecular weight values measured for dopamine β-hydroxylase (chromomembrin A), as the mobility of this protein is markedly affected by reduction (Plate 2.3.). It is not, at present, clear whether the considerable increase in mobility brought about by dithiothreitol-treatment reflects a dissociation of the unreduced protein, giving rise to two identical (or, at least, electrophoretically non-separable) sub-units, or whether it merely results from a large change in conformation, brought about by the breakage of intrachain disulphide bonds (or even, indeed, to an increase in the molar ratio of bound SDS/protein). The native enzyme was reported to have a molecular weight of 290,000 in dilute aqueous buffer (Friedman & Kaufman, 1965) and this has been confirmed in a recent study (Foldes, Jeffrey, Preston & Austin, 1973), in which the weight-average molecular weight was found to be 293,800 in 10mM phosphate buffer (pH 7.2) containing 0.1M sodium chloride. The apparent molecular weight of 135,500 recorded for unreduced chromomembrin A in 6% gels (Table 2.2) is not obviously related to the molecular weight of the native enzyme, since it lies between one half and one third of 290,000. However, the average molecular weight found for reduced samples of

rechromatographed chromomembrin A was 73,790 (compared with the component with a molecular weight of 73,543 seen in the pattern from whole membranes after reduction - see: Table 2.3) and this corresponds with a calculated value of 73,450 which is based on the assumption that the native enzyme is broken down into four identical sub-units under these conditions (taking the molecular weight value of Foldes et al., 1973).

The agreement between these two figures is close and encourages the idea that dopamine β-hydroxylase is a tetrameric enzyme, which is contrary to the conclusion of Foldes et al., (1973), who found a sub-unit of molecular weight 97,000 (estimated by ultracentrifugal methods) to be formed on exposure to 0.1% SDS without reducing agent, and who reasoned that this showed the native enzyme to be a trimer. It is not clear whether protein-bound SDS was allowed for when calculating this (97,000) molecular weight and, since their claim is not easily reconciled with my results, it is unfortunate that Foldes et al., (1973) did not examine the effect of dithiothreitol on their 97,000 molecular weight component. It seems probable that unreduced chromomembrin A, which I observe to migrate electrophoretically as if it had a molecular weight of 135,500, is not a dimer of the 73,500 molecular weight sub-unit (since the mobility of the unreduced protein should give an overestimate, rather than an underestimate, of the expected value of 147,000) but is, instead, a form with a different

conformation which may possibly bind less SDS than the reduced protein and which is retarded more strongly by the gel matrix than globular proteins of equivalent size. If this is so, it does not necessarily follow that sedimentation in the ultracentrifuge will give the same molecular weight estimate as the electrophoretic method.

It was noted earlier that the most commonly-employed method of determining molecular weights by electrophoresis, which uses a calibration graph of log (molecular weight) versus R_m x 100, is empirical and that the preferred method, which can be justified on theoretical grounds (Neville, 1971), makes use of a calibration graph of log (R_m x 100) versus molecular weight. However, when applied to chromaffin granule membrane proteins run in 6% gels, this latter method was found (see: Table 2.4) to give results virtually identical with those obtained in the standard way, and there is, in this instance, no reason for preferring the less widely-accepted way of plotting the calibration graph.

Gel-filtration on a calibrated column offers an alternative route to the determination of molecular weights, if sufficient sample is available, and so this method was also applied to unreduced chromaffin granule membrane proteins (Table 3.1). The resolution of proteins on the gel-filtration column is much less than can be achieved in electrophoretic separations and the molecular weight estimates are therefore more liable to error;

however, a good correspondence was observed between the molecular weights found by gel-filtration and those determined electrophoretically. Thus, unreduced chromomembrin A was found to behave as if it had a molecular weight of 135,680 (mean of three determinations, standard error of the mean = 266) - a figure remarkably close to the value of 135,500 derived from the electrophoretic experiments. The mean molecular weights calculated for chromogranin A (component C) and chromomembrin B were slightly higher than the electrophoretically-measured figures, being 90,785 ($\pm$ 2515, mean of 2) versus 80,319 and 31,623 ($\pm$ 1547, mean of 3) versus 28,728 respectively, but these differences may arise from errors in constructing the gel-filtration calibration graph, since a much smaller number of data points were used when calculating this regression line than were used for the electrophoretic calibration graphs. While this good agreement between two sets of molecular weight-determination experiments is gratifying from the technical point of view, the physical processes governing separation in gel-filtration and gel-electrophoresis are rather similar, and these two methods should perhaps be regarded as being complementary rather than independent.

Quantitative Densitometry.

The extent to which chromaffin granule membrane preparations are still contaminated by the soluble vesicle proteins has, for some time, been a source of controversy between the Innsbruck group and Helle, Winkler et al., (1970) reporting that their membrane preparation

contained ca. 1% of chromogranin A, which comprises 38% of the soluble protein (Smith & Winkler, 1967b), while Helle has claimed chromogranin A contents of 73-75%, 15-35% and 21% for various membrane samples, prepared by differing methods. My quantitative densitometry experiments suggest that the measurements of Winkler et al., (1970), whose preparative method I used, are erroneously low, most probably because of inaccessibility of part of the membrane-associated chromogranin A to its specific antibody (used for its quantitation by micro-complement fixation), and that a better estimate for the amount of protein present in the membrane as chromogranin A is 13%. By contrast, the chromogranin A content of a membrane sample prepared by the dialysis method of Helle (1971) was found to be 18%, and this is in relatively good agreement with her latest claims, which suggests, in turn, that this dialysis method is rather less effective in removing soluble proteins from the chromaffin granules than the cycles of freezing and thawing used by Winkler et al., (1970). Furthermore, the membranes prepared by Helle's method appear to contain more of the component designated "F" in Fig. 2.7 (see: Fig. 2.6a) and it may be that this component is associated in some way with chromogranin A.

The results of the quantitative densitometry experiment must, however, be interpreted with caution, since the experimental procedure itself is not of very high precision and since the calculation of the percentage of the total membrane protein which is

chromogranin A makes an important assumption. This assumption is that the relative intensity of staining of chromogranin A (i.e.: mg dye bound per mg of protein measured by the Folin method) is similar to that of other membrane proteins present in the sample. Winkler et al., (1970) have suggested, but not demonstrated, that this is not true, and that chromogranin A is stained very intensely (on a weight basis) by Amido Black. If this is so, and a comparison with the staining intensities of known amounts of standard proteins such as ovalbumin suggests that it may be, it would inflate the estimate of the percentage of chromogranin A in both membrane preparations, without affecting the conclusion that relatively more is present in the membrane sample prepared by Helle's method.

If the densitometric evidence that 13% of the membrane protein is chromogranin A is correct, it may be calculated that, if there is no preferential binding of this protein relative to other soluble lysate proteins, then ca. 30% of the protein in the membrane pellet is derived from adsorbed soluble proteins (since chromogranin A makes up 38% of the soluble protein). However, the amino acid analysis of total soluble proteins shows a characteristically high content (20%) of glutamate (Strieder, Ziegler, Winkler & Smith, 1968) which is not so pronounced in the analysis of the total membranes (which contain 15.4% by weight glutamate - see Winkler et et al., 1970) and Winkler et al., (1970) showed that the amino acid composition of the membrane fraction was not significantly altered

by trypsin digestion, which caused a 31% reduction in protein content and specifically reduced both the immunological titre of chromogranin A in the membranes and the staining intensity of the chromogranin A band in the electrophoretic pattern, in line with the expectation that chromogranin A is readily digested by trypsin (Smith & Kirshner, 1967). In view of this conflicting evidence, it is not possible to reach a firm conclusion about the extent of contamination of the membrane fraction by soluble proteins, although the observation that many bands present in the phosphate/SDS gel pattern of soluble lysate proteins are also visible, as minor components, in the pattern from solubilised membranes may indicate that this cross-contamination is more significant than Winkler et al., (1970) have suggested.

The Gel-filtration Experiments.

The very earliest gel-filtration experiments I performed, with the aim of preparing purified chromomembrin A and chromomembrin B for chemical analysis, made use of the borate buffer/SDS/Sephadex G-200 methodology described by Hörtnagl et al., (1971). Except for the use of a slightly shorter and rather broader column, the conditions of the experiments were kept as close as possible to those described by the Innsbruck group and I was, therefore, rather surprised by the difference in elution profiles (Fig. 3.1), particularly the discrepancy in the position of elution of the phospholipids. With the use of the improved electrophoretic system it became evident that the peak-separation observed in these

experiments was not sufficiently great to give adequate supplies of pure proteins for further study, and I therefore decided to try to find a gel-filtration system with higher resolution.

This requirement was found to be satisfied by gel-filtration on a bed of Sepharose 6B, using a tris/HCl buffer of less alkaline pH and containing 0.1% SDS, and this system was used preparatively to obtain quantities of purified chromomembrin A, chromomembrin B and membrane cytochrome. However, even with this system, the phospholipids were not separated from the final peak of protein and since these samples were shown by electrophoresis not to be homogeneous, each purified fraction was submitted to a second cycle of chromatography, using the same column.

Even after rechromatography, however, the protein preparations were not free of impurities and one of them, the cytochrome, appeared to have been dissociated by prolonged exposure to SDS. Since this gel-filtration regime was more rigorous than that described by Hörtnagl et al., (1971) and yet still did not yield pure products, it would seem likely that their "purified" chromomembrin A and chromomembrin B contain contaminants which were not detected by their electrophoretic analysis procedure. This idea is supported by their claim to be able to separate two sub-units of reduced chromomembrin A by gel electrophoresis, whereas I find only one band to be produced by reduction of the (soluble) dopamine β-hydroxylase and conclude that "chromomembrin A_2" is a contaminant protein rather than a true

sub-unit. In a recent personal letter, Winkler has stated that he has confirmed this result with soluble dopamine β-hydroxylase and now considers "chromomembrin A_2" to be an artifact. In the same letter, he notes that he has also come to the conclusion that the membrane cytochrome may be dissociated into heme and apoprotein moieties by SDS, during the gel-filtration.

Alternative Purification Methods.

It is apparent from the results of the studies described in the previous chapter that it did not prove possible to obtain truly pure preparations of any of the chromaffin granule membrane proteins, and it seems unlikely that the technique of gel-filtration in SDS-containing buffers is capable of extension and modification to provide material of sufficient homogeneity for chemical studies of its structure and composition to be worthwhile. In view of this unencouraging conclusion, what other methods might be used to achieve a greater degree of purification?

Starting with the sample solution of SDS-solubilised chromaffin granule membranes, only preparative polyacrylamide gel electrophoresis would appear to offer the possibility of better resolution. However, there are disadvantages to this technique, since the amount of protein that can be loaded onto a gel is severely limited if high resolution is desired (as would be necessary for the separation of chromaffin granule membrane proteins) and some proteins may show low recoveries from the gel (Kapadia & Chrambach, 1972). Since the

phosphate/SDS buffer has a high electrical conductivity, which leads to a low voltage gradient across the separating gel, it is likely that a preparative separation would be very prolonged, when the same problem of dissociation of the membrane cytochrome would be encountered as was found with gel-filtration. Furthermore, this purification procedure still yields a protein product which has been denatured by exposure to SDS, and will therefore have lost many of the structural and enzymic properties essential for its function in the intact membrane.

Since this is so, the most productive approach to the study of the structures and functions of chromaffin granule membrane proteins might be to try to isolate them in an undenatured state, by careful extraction of the membrane pellet with non-denaturing reagents, such as dilute buffer solutions, chelating agents (e.g.: EDTA) mild organic solvents and nonionic detergents, perhaps using each of these in turn, to bring about a controlled release of different classes of protein from the membranes. Any "core" of proteins resistant to this sequence of reagents might be solubilised by digestion of the remaining phospholipids with phospholipases, followed by lysophospholipases (van den Bosch & Poorthuis, 1972) - a procedure which has been shown to result in almost complete removal of phospholipids from subcellular membranes. If such an approach were followed, it might be possible to use some of the powerful separative methods, such as ion-exchange chromatography,

which are not applicable in the presence of ionic detergents such as SDS.

Prospect.

Since it is likely to prove very difficult to isolate and purify the various proteins of chromaffin granule membranes, particularly those which are only minor components of the membrane, it is worthwhile considering in what ways biochemical and chemical studies of these proteins may be expected to contribute to our understanding of the life-cycle and functioning of the chromaffin granule, and, in particular, to the elucidation of the mechanism of exocytosis by which the granules release their stores of amine, nucleotide and protein from the chromaffin cell.

In addition to dopamine β-hydroxylase, Mg^{++}-activated ATPase and cytochrome b_{559}, chromaffin granules have been reported to contain a phosphatidylinositol kinase activity (Buckley, Lefebvre & Hawthorne, 1971), one or more flavoproteins and a NADH; cytochrome oxido-reductase activity (Flatmark, Terland & Helle, 1971), the latter components probably forming part of an electron-transport chain, linked to dopamine β-hydroxylase. The membrane of the chromaffin granule has a low content of protein relative to lipid (Winkler et al., 1970) and it is to be expected that there will be little "structural" protein (that is, protein without an apparent role to play, other than in maintaining the morphological integrity of the organelle), whose presence would in any case be undesirable,

since the chromaffin granule must very probably possess a more flexible membrane than other cell particles because of the requirement that it must fuse with the cell membrane during exocytosis, and it is also possible that the (rather limited number of) membrane proteins may each regulate more than one of the processes occurring during the life cycle of the granule, or at least that some of these processes may be interdependent. Thus, the hydrolysis of ATP by the membrane Mg^{++}-ATPase which has been shown to drive catecholamine uptake (Taugner, 1971) results in the phosphorylation of both membrane protein (whose nature is at present unknown) and membrane lipids, the diphosphatidyl inositol fraction showing the largest incorporation of phosphate (Trifaró, 1972; Buckley et al., 1971).

Chromaffin granules may be regarded as providing (because of their relative structural and chemical simplicity) the most amenable naturally-occurring system for the study of those biophysical processes peculiarto entities with a degree of organisational complexity greater than that shown by soluble enzymes. The distinguishing features of this kind of system are that its components are related to one another through their topographical arrangement, which is likely to bear closely on their functions, and that the various constituents are likely to be "compartmentalised" by the presence of diffusion-barriers. The membrane of the chromaffin granule appears to be the site at which most of the enzymatic reactions take place, and it is an unfortunate fact that virtually nothing is known about the

arrangement of the various proteins in this membrane. Numerous 8.5 - 10nm particles have been seen projecting from the chromaffin granule membrane in freeze-fracture studies in the electron-microscope (Smith, Smith, Winkler & Ryan, 1973), but it is not known whether chromomembrin A and chromomembrin B are confined to one or other face of the membrane, or whether they penetrate through it.

A large number of investigations have been carried out using erythrocytes (whose membranes have a very much more complicated protein composition than chromaffin granule membranes) in which the principal aim has been to determine the distribution of various proteins between the inside and outside faces of the membrane, and many of the techniques used in this field may be expected to yield fruitful results when applied to chromaffin granules. Thus, lactoperoxidase-catalysed iodination (Phillips & Morrison, 1971), reaction with membrane-impermeable reagents such as S-formyl-methionyl sulphone methyl phosphate (Bretscher, 1971) and diazotized sulphanilic acid (Carraway, Kobylka & Triplett, 1971) and digestion with proteases (Triplett & Carraway, 1972; Steck, Fairbanks & Wallach, 1971) have all been used to demonstrate particular proteins to be localised on one or other side of the membrane. Differential labelling of the phospholipid phosphatidyl-ethanolamine has also been reported (Bretscher, 1972) and it should not be assumed that the distribution of lipid between the two faces of the membrane is

necessarily uniform. Evidence has also been found that the major glycoprotein of the erythrocyte membrane penetrates right through the membrane (Bretscher, 1971) and the amino acid sequence of the postulated intramembranous region has been determined (Segrest, Jackson, Marchesi, Guyer & Terry, 1972).

Information about the disposition of the membrane proteins of the chromaffin granule which might be gained through the application of these techniques is especially valuable, since it may shed some light on the biochemical mechanism of exocytosis. This is again a field in which experimentally-established facts are not plentiful, and a variety of mechanisms have been proposed for the fusion process. Any such model of this mechanism must take into account the natures and properties of both chromaffin granule and plasma membranes, since the process of exocytosis involves the fusion of two membranes. Experimental studies are hampered by the lack of a pure preparation of the plasma membrane, which might be used to study the interaction directly. Among the suggestions advanced for the mechanism of this interaction, is the idea that microtubules may be involved in the release process (Poisner & Bernstein, 1971), since it was found that the release of catecholamines from perfused medulla was inhibited by colchicine and vinblastine, which are known to disaggregate microtubules. The microtubular sub-unit protein Tubulin was isolated from the supernatant of homogenates of bovine adrenal medullae by Redburn, Poisner &

Samson (1972), which appeared to add plausibility to this model, but it was later shown (Trifaró, Collier, Lastowecka & Stern, 1972) that the inhibition of catecholamine release from the perfused medulla brought about by colchicine and vinblastine was due to these drugs acting as antagonists to acetylcholine and nicotine, used to stimulate the gland. It is interesting, however, that in one electron-micrograph published by Benedeczky & Smith (1972 - see: Fig. 4 and inset) cross-bridges can be observed between chromaffin granule and the plasma membrane.

In conclusion, it appears that much remains to be learned about the nature of the chromaffin granule and the mechanism of the exocytotic process. Biochemical interest in this system need not, therefore, stop short with the problems of the storage of the neurotransmitter (which is still poorly understood) and the achievement of the elucidation of the biosynthetic and degradative pathways of catecholamine metabolism, but may be much stimulated by the application of biophysical and related techniques to the study of these systems and, together with the very important pharmacological and physiological investigations of adrenal medullary and sympathetic nervous tissue, may at last lead to a satisfactory understanding of the molecular mechanism of nerve-conduction in the sympathetic nervous system.

CHAPTER 5.

Electrophoretic Studies of the Proteins of Noradrenergic Vesicles from Bovine Splenic Nerve Axons.

Methods 99

Results

The soluble proteins of fractions FI, FII and FIII. . . 102

The insoluble proteins of the splenic nerve fractions . 106

Discussion 109

METHODS

The preparations enriched in noradrenergic vesicles from bovine splenic nerves used in these studies were kindly made available by Dr. H. Lagercrantz of the Department of Physiology I, Karolinska Institutet, Stockholm, Sweden. The preparations were made in Stockholm, following the method described by Lagercrantz, Klein & Stjärne (1970), and were deep-frozen for transport to Oxford. The preparative methods and characterisation of the various fractions have been reported in detail (Lagercrantz et al., 1970; see also: Lagercrantz, 1971 and Klein & Lagercrantz, 1971), but the following brief description is included for ease of identification of the various subfractions examined in my study.

Homogenates of bovine splenic nerves in 0.25M sucrose were centrifuged for 10 minutes at $600g_{max}$ to remove coarse debris and the resulting supernatant was filtered through gauze, before being centrifuged at $10,500g_{max}$ for 10 minutes (which removes a large proportion of the mitochondria from the suspension, without excessive sedimentation of the noradrenergic vesicles). The supernatant from this centrifugation was layered onto linear density-gradients, ranging from 0.25M sucrose in 50% H_2O/50% D_2O to 1.5M sucrose in 100% D_2O, and the gradients were then centrifuged for 90 minutes at 40,000 r.p.m. ($100,000g_{max}$) in the SW40 rotor of a Beckman L2-65B ultracentrifuge. After centrifugation, the

three opaque bands visible in each tube (denoted FI, FII and FIII, in order of increasing density) were withdrawn and diluted to isotonicity and centrifuged in a Ti50 rotor at 226,000g_{max} for 30 minutes, to give three pelleted fractions. For one experiment, fractions FII and FIII were combined and re-centrifuged on a second gradient (under identical conditions to the first density-gradient centrifugation) to give repurified fractions, denoted RII and RIII (with buoyant densities corresponding to those of FII and FIII respectively).

After transportation to Oxford, the fractions were allowed to thaw and were then "lysed" by suspension in 0.005M tris-sodium succinate buffer (pH 5.9), using 5.0ml of buffer per fraction (which was usually the pooled material from three density-gradients), followed by freezing in the deep-freeze, thawing and centrifugation (at 30,000 r.p.m., (59,360g_{av}) in the 40 rotor of the ultracentrifuge, for 60 minutes) exactly as described in an earlier chapter for chromaffin granules. This entire lysis procedure was repeated twice more, yielding (for each sample) a final ("membrane") pellet and three supernatants. The first and second of these supernatants were combined and ultrafiltered at 2° to a volume of ca. 0.5ml and this preparation will be referred to simply as "soluble proteins".

The membrane pellets were each dissolved, with heating, by the addition of ca. 1ml of 1% SDS, 10% sucrose in 0.01M sodium phosphate buffer (pH 6.5), exactly as for chromaffin granule

membranes. The soluble protein solutions were made dense by the addition, to each, of a small quantity of solid sucrose. The electrophoretic analyses of both the soluble proteins (with tris-glycine buffers) and the SDS-dissolved membrane proteins (with 0.1% SDS/0.1M sodium phosphate buffer, pH 6.5) were carried out in 6% polyacrylamide gels under conditions identical to those described for chromaffin granule proteins.

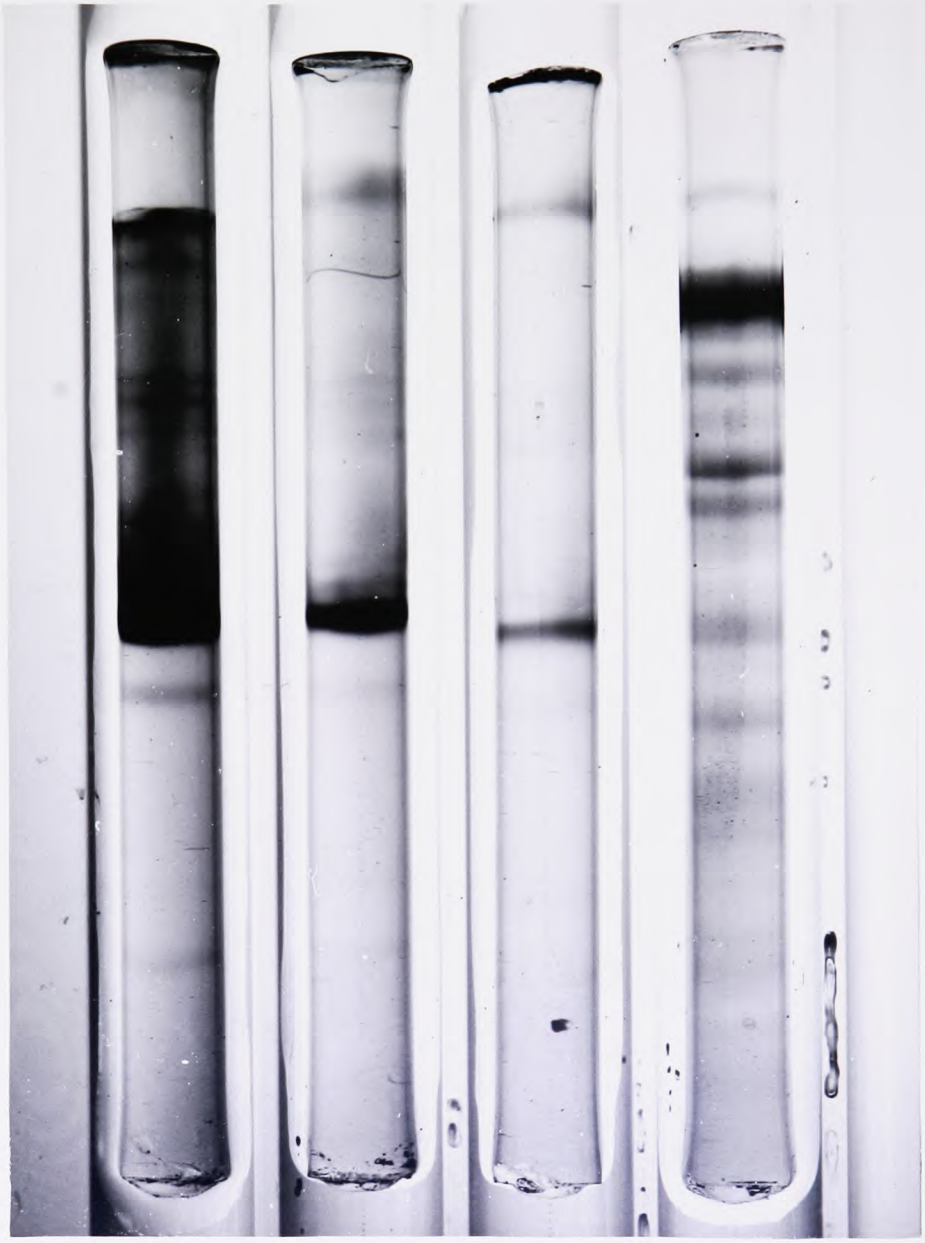


Plate 5.1. Electrophoretic Patterns of the Soluble Proteins from Bovine Splenic Nerve Fractions.

Electrophoresis in 6% polyacrylamide gels was carried out with the tris/glycine buffer system. The samples applied were (from left to right): soluble proteins from fractions FI, FII and FIII and (for comparison) bovine adrenal chromaffin granules.

RESULTS

The Soluble Proteins of Fractions FI, FII and FIII.

Several workers have described experiments which suggest that dopamine β-hydroxylase and chromogranin A are present in the noradrenergic vesicles of bovine splenic nerves (Hörtnagl, Hörtnagl & Winkler, 1969; Banks, Helle & Mayor, 1969 and De Potter, Smith & De Schaepdryver, 1970), and one of my first objectives was to try to demonstrate these two proteins in the "soluble proteins" of the gradient fractions, by the visualisation of stained bands with their characteristic relative mobilities in polyacrylamide gels run with the tris-glycine buffer used for studies of the soluble proteins from bovine adrenal medullary chromaffin granules (chromogranins). The chromogranins give a well-defined and reproducible pattern with this buffer system and their relative mobilities had already been measured (S.F. Bartlett, unpublished observations) and were therefore available for comparison.

The staining patterns of the "soluble proteins" of fractions FI, FII and FIII (see: Plate 5.1) showed several differences from the typical chromogranin pattern. Firstly, the most prominent component in all three fractions was observed to have a mobility of $R_m \times 100 = 51.9$, whereas chromogranin A, with a mobility of $R_m \times 100 = 18 - 20$, is the most intensely-stained band in the chromogranin pattern. Secondly, although the bands themselves were quite sharply-defined, there was a diffuse background of

staining, extending from the origin to this major band. This diffuse staining, not normally noted with other soluble protein preparations, was always evident in samples of splenic nerve material and its cause is unknown.

From the concentration of noradrenaline per milligramme of protein in each of the fractions, Lagercrantz had concluded that FIII was the fraction which showed the greatest enrichment in noradrenergic vesicles, and it was therefore expected that the soluble protein pattern from FIII would show the strongest bands of dopamine β-hydroxylase and chromogranin A. However, the intensity of staining of the band with a relative mobility close to that expected for dopamine β-hydroxylase was approximately the same in gels from both of these fractions. Further, only faint traces of a band with the mobility of chromogranin A were visible in gels from either FIII or FII, the staining being perhaps marginally greater in FII (although this is, unfortunately, not well shown by photography). In this context, it is interesting that although Lagercrantz (1971) has clearly shown that fraction FII contains about 50% of the "chromogranin A" (identified and quantitated by micro-complement fixation, using antiserum to purified chromogranin A) and 21% of the noradrenaline applied to the density-gradient, while fraction FIII contains 32% of the chromogranin A and 38% of the noradrenaline (values calculated from Fig. 1 of Lagercrantz, 1971), he nevertheless comments

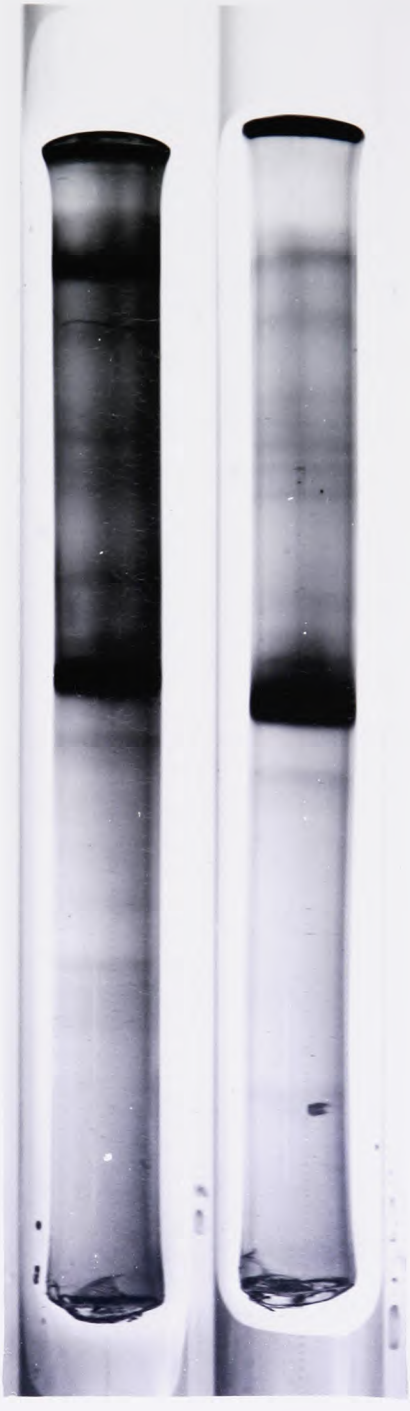


Plate 5.2. Electrophoretic Patterns of Soluble Proteins from Myelin and Myelin Vesicle Fractions.

Electrophoretic conditions were as described in the legend to Plate 5.1., and the samples were from fraction RII (left) and fraction RIII (right).

(ibid., p.20): "The subcellular distribution of chromogranin A in the density gradient fractions of the nerve trunk vesicles preparation was about the same as that of noradrenaline. Is it, in fact, possible that fraction FII contains a larger proportion of chromogranin-like protein than FIII?

The results of the one experiment with re-purified fractions (RII and RIII) may suggest that this is the case. As may be seen from Plate 5.2, the soluble protein from RII contains a much more prominent band with the mobility of dopamine β-hydroxylase than does the soluble protein from RIII. Interestingly, the putative "chromogranin A" band remains very faint in both of these gels.

What is the origin of the very strongly-stained band with a relative mobility of $R_m \times 100 = 51.9$ which is present in all of these fractions? A band with the same mobility is also seen in electrophoretic patterns of the high-speed (particle-free) supernatant ("adrenal SNIII") prepared from homogenates of bovine adrenal medullae (by centrifugation of the "medium-speed supernatant" at 35,000 r.p.m., ($= 80,790 g_{av}$) for 60 minutes - see also under "preparation of chromaffin granules" in chapter 2) and there are similarities between the electrophoretic patterns of adrenal SNIII and the soluble proteins of the splenic nerve fractions. Adrenal SNIII almost certainly contains some contaminating blood proteins and would, therefore, be expected to contain bovine serum albumin, which has a relative mobility of 49.63 (mean of 8 determinations,

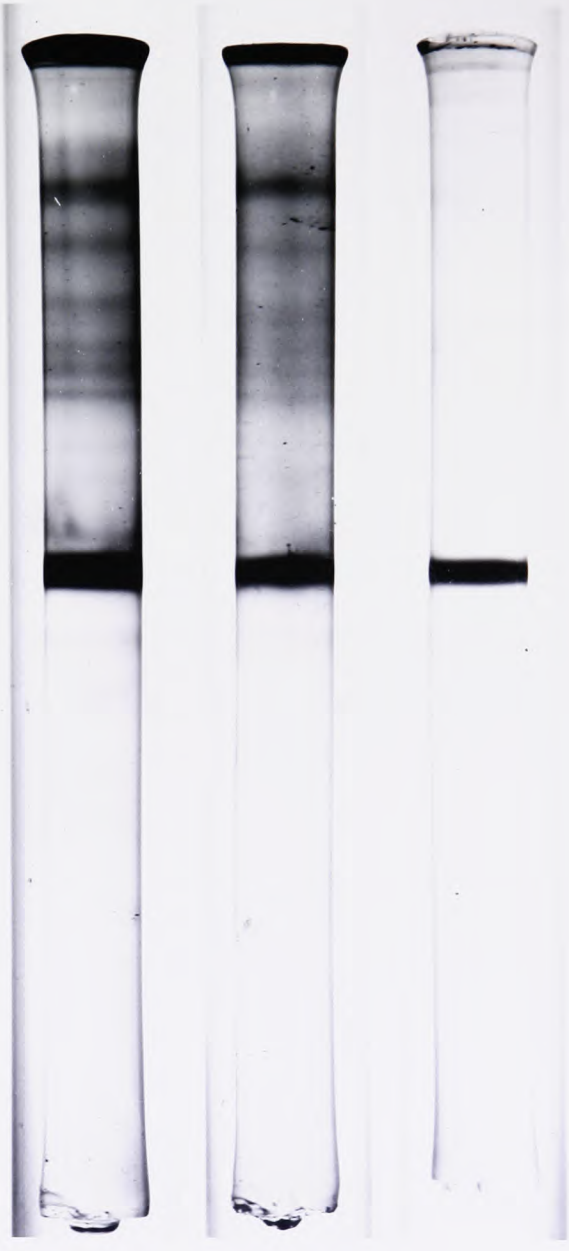


Plate 5.3. Comparison of Electrophoretic Patterns of Fractions FII and FIII with Bovine Serum Proteins.

The electrophoretic conditions were as described in the legend to Plate 5.1., and the samples were (from left to right): fraction FII, fraction FIII, diluted bovine serum.

SEM = 0.101) under these electrophoretic conditions. Bovine serum albumin stains very strongly with Amido Black and it is reasonable to suppose that the major band of the adrenal SNIII pattern represents contaminating bovine serum albumin. Is it likely, therefore, that the major band of the "soluble protein" patterns of FII and FIII, and possibly other minor components too, represent adsorbed blood proteins, rather than proteins associated with the noradrenergic vesicles?

A partial answer to this question is given by the patterns in Plate 5.3, in which gels containing soluble protein from fractions FII and FIII are compared with a sample of diluted bovine serum containing 9 μg of (total) protein. This quantity of serum was chosen to give an albumin band with a staining-intensity as close as possible to that of the major band in FII and FIII. It can be seen that the prominent minor components of the splenic nerve fractions are not visible in the gel of the serum proteins, and it seems likely that these components are not blood proteins. It is, however, entirely possible that the major band in the splenic nerve fractions may be due to the presence of bovine serum albumin in the samples.

Two faint bands with higher mobilities than bovine serum albumin are also visible in the splenic nerve preparations, but are absent (even at high loadings) from the patterns given by bovine serum. These two bands faded rather quickly on destaining

Table 5.1

Relative Mobilities and Staining Intensities of Soluble Proteins from Bovine Splenic Nerve Fractions FII and FIII.

Fraction FII			Fraction FIII		
Mean R_m x 100	SEM (No.)	Staining	Mean R_m x 100	SEM (No.)	Staining
12.13	0.797 (5)	F/M	12.59	0.785 (5)	F/M
19.45	0.705 (4)	VF	19.99	0.592 (3)	VF
25.63	0.623 (4)	VF	26.72	0.723 (3)	VF
31.24	0.759 (4)	VF			
34.46	0.676 (3)	VF	34.08	0.349 (3)	VF
			36.58	0.995 (2)	VF
43.35	0.465 (2)	VF	43.80	0.993 (3)	VF
51.57	0.525 (2)	VF	49.09	0.583 (3)	VF
51.93	1.140 (5)	VS	51.93	1.102 (5)	VS
60.89	1.204 (3)	F	60.98	1.115 (4)	F
91.10	0.901 (3)	VF	91.67	0.455 (2)	VF

Key to abbreviations:

F: faint, M: medium, VF: very faint, VS: very strong.

All measurements were made in 6% polyacrylamide gels, run with the tris-glycine buffers of Clarke (1964).

and did not photograph well, but they were, nonetheless, reproducible components of the splenic nerve patterns. Minor bands with similar mobilities were also noted in the patterns from adrenal chromogranins and adrenal SNIII and it is possible that these components (visible in the splenic nerve gels) are associated with either neurosecretory vesicles or some other structure found in both tissues, for example: microtubules (Wood, 1967; Redburn, Poisner & Samson, 1972) - which are very easily disrupted during subcellular fractionation. The relative mobilities and staining intensities of the components of FII and FIII detected by electrophoresis with the tris-glycine buffer system are set out in Table 5.1.

The Insoluble Proteins of the Splenic Nerve Fractions.

The SDS-dissolved insoluble ("membrane") proteins of the splenic nerve fractions gave much more complicated patterns on electrophoresis with the phosphate-SDS buffer system than did similarly-treated chromaffin granule membrane proteins. Both FII and FIII gave patterns containing more than twenty distinguishable components and gels representative of each are shown in Plate 5.4, the bands with mobilities greater than $R_m \times 100 =$ ca. 50 being a little distorted in the FII gel illustrated. Although the band-patterns were usually well-formed, it was found that these samples were particularly likely to produce artifacts, such as band-distortion or a spurious ring around the outside of the gel - caused by shrinkage of the gel away from the glass running-tube, and this may have been a consequence

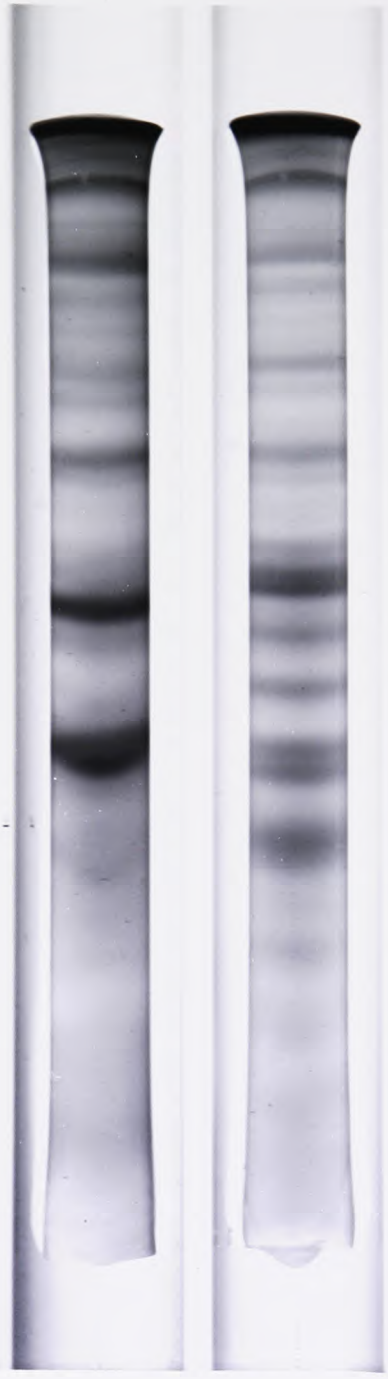


Plate 5.4. Electrophoretic Patterns of the Insoluble Proteins from Bovine Splenic Nerve Fractions.

Electrophoresis in 6% polyacrylamide gels was carried out with the phosphate/SDS buffer system. The samples applied were: fraction FII (left) and fraction FIII (right).

Table 5.2

Relative Mobilities and Staining Intensities of SDS-solubilised Membrane Proteins from Bovine Splenic Nerve Fraction FII and FIII in 6% Polyacrylamide Gels (Phosphate/SDS buffer).

Fraction FII.				Fraction FIII.			
Mean R_m x 100.	SEM.	No.	Staining.	Mean R_m x 100.	SEM.	No.	Staining.
3.35	0.536	3	F	4.50	0.088	6	F
6.58	0.309	4	F/M	7.28	0.304	8	F/M
9.14	0.383	3	F				
13.71	0.142	6	F	13.74	0.160	7	VF
				15.87	0.350	6	VF
17.33	0.393	6	F	18.88	0.260	7	F
20.14	0.312	6	VF	21.55	0.373	6	VF
23.96	0.236	3	F	25.62	0.287	8	F/M
26.27	0.323	6	F	28.18	0.284	7	VF
29.41	0.280	2	VF	30.32	0.582	3	VF
34.69	0.486	6	F/M	34.37	0.307	9	F
				36.94	0.411	6	VF
				40.01	0.291	3	VF
				43.14	0.354	6	F/M
45.92	0.669	4	VF	46.01	0.281	6	M
48.63	0.357	6	S	48.52	0.336	9	S
52.95	0.652	6	F	53.16	0.377	9	M
57.67	0.604	5	F	58.38	0.345	9	F/M
63.11	0.453	5	M/S	64.30	0.327	7	F/M
66.82	0.309	3	F	67.27	0.255	9	F/M
71.92	0.400	3	F				
74.75	0.332	4	F	73.44	0.426	9	M
79.43	1.145	2	VF	81.41	0.275	2	VF
86.03	0.207	3	VF	85.19	0.561	8	F
				91.62	0.545	7	F
				97.69	0.358	4	F

Key to abbreviations: VF: very faint, F: faint, M: medium, S: strong.

of the fact that there was always material which did not enter the gel, as shown by the heavy staining of the origin.

The relative mobilities and staining intensities of the insoluble proteins of FII and FIII are set out together in Table 5.2, If protein from neurosecretory vesicles made up the major part of the membranous protein present in these fractions, and if neurosecretory vesicle membranes have a similar protein composition to chromaffin granule membranes (which is, as yet, an unproven assumption), then there should be an obvious correspondence between the major bands of the FIII (or FII?) pattern and those of chromaffin granule membrane patterns (see Chapter 2). Although there are a number of near correspondences in relative mobilities between FIII bands and chromomembrins, the presence of a large number of bands in the splenic nerve gels makes it difficult to assert the identity of nerve and adrenal proteins with any degree of confidence. Once again, it seems likely that the most intensely-stained band is due to bovine serum albumin and possible "chromogranin-like" components in the pattern are of minor staining intensity. Most strikingly, only a very faint band appears in the region where chromogranin A would be expected to be localised ($R_m \times 100 = 36 - 38$).

The membranous proteins of the re-purified fractions RII and RIII were also examined in the phosphate-SDS system and the patterns obtained with these preparations are shown in Plate 5.5 (the gel

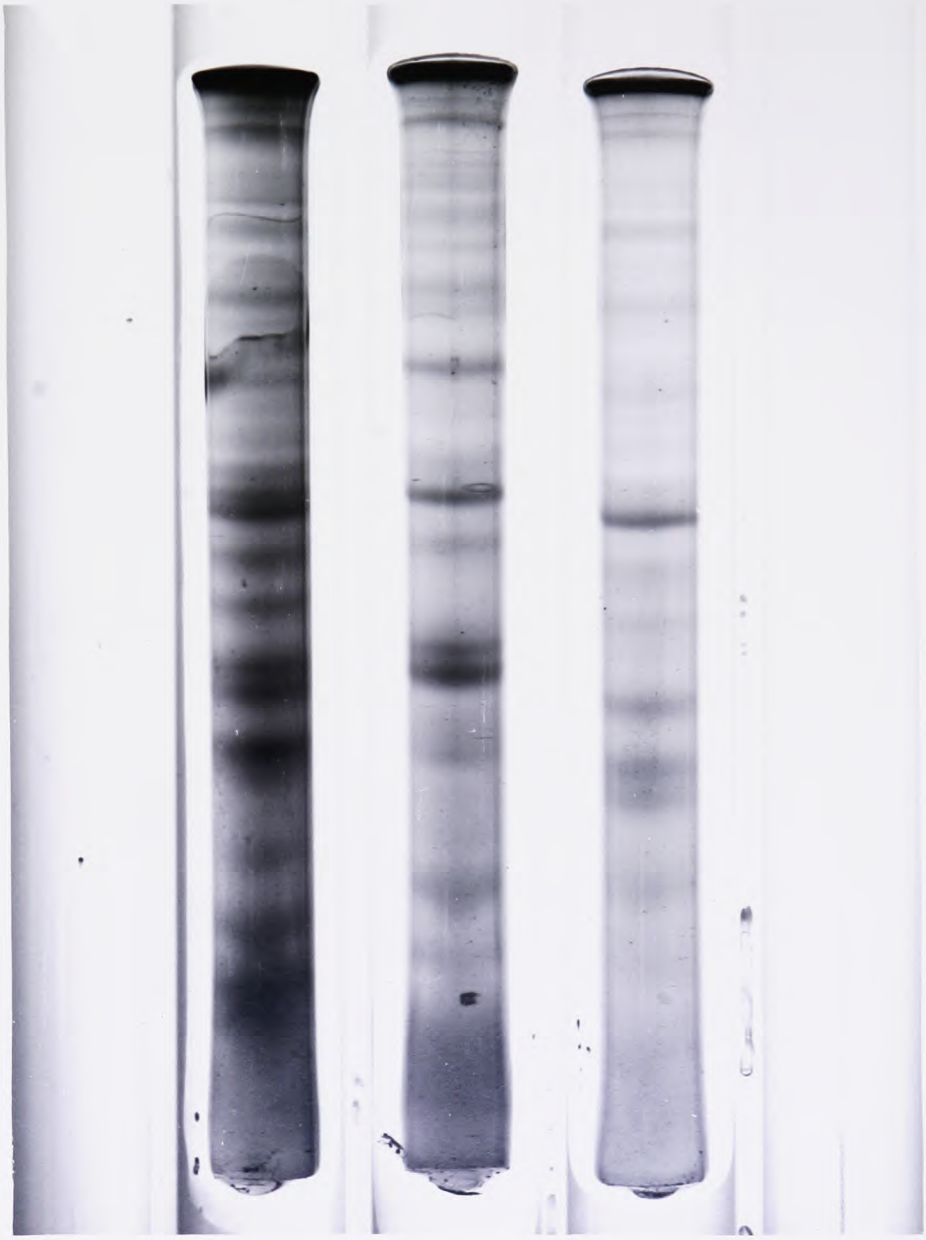


Plate 5.5. Electrophoretic Patterns of the Insoluble Proteins from Repurified Nerve Vesicle Fractions.

Electrophoresis was carried out as described in the legend to Plate 5.4. The samples were (left to right): fraction FIII, fraction RII and fraction RI.

Table 5.3

Relative Mobilities and Staining Intensities of SDS-solubilised Membrane Proteins from Re-purified Bovine Splenic Nerve Fractions RII and RIII and Chromaffin Granules (6% gels)

Fraction RII.				Fraction RIII.				Chromaffin Granules.	
Mean R_m x 100	SEM	No.	Staining	Mean R_m x 100	SEM	No.	Staining	Mean R_m x 100	Staining
3.59	0.390	2	F	3.92	0.040	2	F		
5.99	0.375	2	M	6.15	0.395	2	F	5.792	F
15.96	0.140	2	F	16.31	0.450	2	F	18.510	S
19.26	0.025	2	VF	19.53	0.300	2	VF		
23.65	0.215	2	VF	24.16	0.370	2	F		
26.44	0.400	2	VF	26.78	-	1	VF	25.719	F
32.03	0.385	2	M	33.62	0.310	2	F	32.370	F
36.65	-	1	VF	38.95	0.290	2	F	38.329	S
41.81	0.345	2	F	42.78	0.145	2	F	43.777	M
45.69	0.430	2	S	46.40	0.200	2	S	48.583	F
50.48	0.210	2	F/M	51.73	0.020	2	F		
				57.07	0.160	2	F	58.702	F
61.16	0.130	2	M/S					63.678	VF
64.35	0.060	2	S	65.62	0.190	2	M/S	66.789	VF
68.84	0.250	2	VF						
72.24	0.265	2	F	71.87	0.085	2	M		
77.53	-	1	VF	75.29	0.065	2	M	77.306	S
85.81	0.715	2	F/M	84.74	0.525	2	F		
94.13	-	1	VF	92.39	0.405	2	F		
				100.33	0.790	2	VF		
				104.24	-	1	VF		

Key to abbreviations: VF: very faint, F: faint, M: medium, S: strong.

Relative mobility values of chromaffin granule membrane proteins are taken from Table 2.2 and corresponding staining intensities from Table 2.3.

of FIII included for reference in this picture exhibits the kind of "ring artifact" mentioned earlier, which does not, however, affect the relative mobilities or staining intensities of bands within the gel matrix). Although there were noticeable changes in the relative prominences of various bands on re-purification, the band-patterns of both fractions are still complex and almost all of the components visible in FII and FIII can still be recorded in RII and RIII. Once again, the virtual absence of a band at the position expected of chromogranin A is a striking feature of the patterns and even the possible dopamine β-hydroxylase (chromomembrin A) bands in the region $R_m \times 100 \approx 16 - 18$ appear very faint. The relative mobilities and staining intensities of the bands in RII and RIII are given in Table 5.3, together with data for chromaffin granule membrane proteins, copied from Table 2.2. In general, the bands of RII and RIII had slightly lower mobilities than their counterparts in FII and FIII.

DISCUSSION

The chemical composition of bovine adrenal chromaffin granules is now relatively well established, since it has been possible for some time to prepare the granules in a high degree of purity and reasonable quantity for direct chemical analysis. By contrast, knowledge of the composition of bovine splenic nerve vesicles is still at a relatively primitive level, since it has not proved possible to isolate homogeneous preparations for analysis. Thus, the evidence for the location of dopamine β-hydroxylase and chromogranin A within splenic nerve vesicles comes not from chemical determinations but from careful studies of the distributions of these and other "markers" after differential and density-gradient centrifugation (Hörtnagl et al., 1969; De Potter et al., 1970).

Originally, Lagercrantz et al. (1970) had claimed that their preparation FIII contained about 18% of the total protein as vesicle protein, and this estimate was based on the distributions of various marker substances amongst the various gradient-fractions. Later, Lagercrantz (1971) modified this claim, on morphological grounds, arguing that probably 40 - 60% of all the particles present in FIII were noradrenergic vesicles. If it is accepted that ca. 50% of the material present in FIII is of neurovesicular origin, and that the vesicles contain chromogranin A which is capable of being released by osmotic shock (De Potter et al. 1970), it would be expected that

chromogranin A should be a prominent component of the electrophoretic pattern of the soluble proteins of FIII. And yet this was not found to be the case. What explanations can be offered for this discrepancy?

It could be argued, since a band with a mobility similar to that of dopamine β-hydroxylase was seen in gels of the soluble proteins of FIII, that the vesicles do not contain chromogranin A, or that they contain much less than chromaffin granules do. All the previous measurements of chromogranin A content have been made by immunochemical methods, such as microcomplement fixation, and it is difficult to be absolutely confident that the antiserum employed is entirely specific for chromogranin A, since the presence of even a small amount of antibody directed against the highly-antigenic dopamine β-hydroxylase will greatly affect the quantity of "chromogranin A" recorded. However, De Potter et al., (1970) went to considerable trouble to purify their antiserum, by using only highly-purified chromogranin A antigen to produce it and by "absorbing" it by incubation with purified dopamine β-hydroxylase before use, and it is reasonable to suppose that they, at least, were actually measuring chromogranin A. They found that splenic nerve vesicles contained about twice as much chromogranin A as chromaffin granules, when the content was expressed in terms of the noradrenaline content, and were relatively deficient in ATP. However, their most striking conclusion was that the nerve vesicles

contain about 60 times as much dopamine β-hydroxylase activity as adrenal chromaffin granules, when expressed in this way (see: Table 2 of De Potter et al., 1970). In view of this result, it is less surprising that dopamine β-hydroxylase could be seen in the soluble protein patterns from FII and FIII while chromogranin A could hardly be detected, although it seems very likely that the estimate of 50% vesicle content for FIII is over-optimistic.

The observation that the dopamine β-hydroxylase band was relatively more intense in the soluble protein pattern of RII than in RIII is also of interest. De Potter et al. (1970) noted that the distribution of this enzyme did not exactly mirror that of noradrenaline (as it should if the two are confined to the same particle), either in differential or density-gradient centrifugation, and they attributed this to the presence of the enzyme in disrupted vesicles or membrane fragments of slightly lower density than the intact vesicles. In the conditions pertaining in Lagercrantz's density-gradient, this might lead to an "enrichment" of FII with dopamine β-hydroxylase, since the empty vesicles would be expected to sediment in this fraction, and this enrichment would explain the "extra" dopamine β-hydroxylase seen in gels of this fraction. This enrichment would be expected to be more evident in the re-purified fractions, since the combined FII + FIII fraction was diluted to a lower sucrose molarity before being placed on the second density-gradient (for the re-purification

step) and this dilution is likely to cause some of the vesicles to become disrupted by osmotic shock.

The insoluble protein patterns yielded rather less useful information than the soluble protein patterns, but they tended to confirm the idea that a very considerable proportion of the material present in FII and FIII was not of neurovesicular origin, but was instead derived from other subcellular particles. However, when truly pure preparations of sympathetic nerve vesicles are eventually isolated it may be expected that interest will centre on the membrane proteins, since, because of the small size of the nerve vesicles - resulting in a small enclosed volume - compared with adrenal chromaffin granules, the membrane phase is even more important to the functioning of the nerve vesicle than is the case for adrenal chromaffin granules.

CHAPTER 6.

Studies of Protein Synthesis in Sympathetic Ganglia

Methods

	Page
In Vitro labelling of ganglion proteins.	113
Extraction of ganglion proteins with SDS	114
Electrophoretic analysis of ganglion extracts.	115
Sectioning of gels after electrophoresis	116
Treatment of the sections.	117
Scintillation-counting and calculation of results.	118

Results

Investigations into the reliability of the analytical procedure used to study protein synthesis in ganglia . . .	120
Labelling and extraction of ganglion proteins	122
Electrophoretic separation of ganglion proteins.	126
Sectioning of polyacrylamide gels	127
Chemical treatment of gel slices	132
Scintillation counting	139
Calculation of profiles	140
Experimental Studies of protein synthesis in rat superior Cervical ganglia: labelling profiles	143
General features of the profiles	143
Detailed features of profiles - section-cutting, slice-thickness and resolution of overlapping bands	144
(a) The effect of slice-thickness on the pattern . . .	145
(b) A theoretical examination of gel-slicing	148
Experiments with ^{3}H-leucine labelling	156

Page

The effect of shortening the time of labelling 158

The influence of physiological state on labelling-experiments with stimulated and denervated ganglia . . . 159

Electrophoresis of rat adrenal chromaffin granule proteins - comparison with superior cervical ganglion staining patterns 163

Discussion

The gel-slicing methodology 165

Labelling-patterns and factors affecting protein synthesis in rat superior cervical ganglia 169

METHODS

In Vitro Labelling of Ganglion Proteins.

Throughout the studies described in this chapter, proteins of the superior cervical ganglion of the rat were radioactively labelled by incubating the ganglia *in vitro* with L-leucine (either ^{14}C(U) or 4,5-^{3}H, both obtained from the Radiochemical Centre, Amersham, Bucks.) following the general method of Paggi & Toschi (1971), which is based on the techniques reported by Larrabee et al. (1963). The labelling procedure was varied in individual experiments, and departures from the usual technique will be noted separately, but the standard conditions were those summarised below.

Superior cervical ganglia, together with their associated pre- and post-ganglionic nerve trunks (ligated with silk thread), were excised from the rat and de-sheathed as described by Larrabee et al. (1963). They were then placed into ice-cold saline for 10-20 minutes before being mounted in a small incubation chamber (total volume ca. 2ml) where they were incubated at 37° with 1.5ml of leucine-free Eagle's Minimum Essential Medium (prepared by G.I.B.C.O., Grand Island, New York, from the formula given by Eagle (1959), with the omission of L-leucine) which was gassed continuously with 5% carbon dioxide/95% oxygen. If the ganglia were to be electrically stimulated, the pre- and post-ganglionic nerve fibres were drawn tightly into glass capillaries containing the stimulating and recording electrodes. The pre-ganglionic fibres could then be stimulated supramaximally

(10 stimuli per second) and post-ganglionic action potentials could be recorded.

After a pre-incubation period, usually lasting for 30 minutes, the contents of the bath were replaced by 1ml of fresh leucine-free medium and the period of radioactive labelling was started by adding 100 microlitres of medium containing 5μC_i of ^{14}C-leucine (equivalent to 15 nanomoles of leucine). The incorporation of radioactive amino acid was usually allowed to continue for a period of 2½ hours. At the end of the labelling-period, the solution in the bath was replaced by 1.5ml of fresh medium containing non-radioactive L-leucine at a final concentration of 0.5μmoles/ml. The medium in the bath was changed twice more, at times of 20 minutes and 40 minutes after the end of the labelling-period, and the ganglia were allowed to remain in contact with this (nonradioactive) leucine-containing medium until a total time of 2 hours had elapsed from the cessation of labelling.

Extraction of Ganglion Proteins with SDS.

At the end of the washing process the ganglia were removed from the incubation apparatus and placed into a small glass homogeniser with a tightly-fitting glass pestle. The ganglia were then homogenised by hand with a solution of 1%(w/v) SDS in 0.01M sodium phosphate buffer pH 6.5 (75 microlitres per ganglion). After homogenisation, the suspension was heated in a bath of boiling water for three minutes and then left to stand overnight at room temperature, before being centrifuged for 1 hour at 130,000g_{av}. The supernatant resulting from

this treatment (termed "SDS- ganglion-extract") was sucked off and deep-frozen.

Both the radioactive labelling and SDS-extraction of the ganglion proteins were carried out by Dr. Paola Paggi and Professor Giovanni Toschi in the Institute of General Physiology, University of Rome. The ganglion extracts were then packed into Thermos flasks, under powdered solid carbon dioxide (dry ice), flown to London and subsequently brought to Oxford for analysis. Throughout their transportation from Rome the samples remained frozen and after arriving in Oxford they were stored in the deep-freeze (at -20^{o}) until they were analysed.

Electrophoretic Analysis of Ganglion Extracts.

Before electrophoretic analysis the ganglion extracts were made dense by the addition of a small quantity of sucrose. Aliquots of 5 or 10 microlitres, used to determine the radioactivity of the sample solution, were placed directly into glass scintillation vials using microcapillaries (Drummond Scientific Company). Quantities of either 20 or 50 microlitres of the extracts were then applied to 6% polyacrylamide gels (containing 0.1%(w/v) SDS and 0.1M sodium phosphate buffer pH 6.5) using either an Eppendorf automatic pipette (Eppendorf Gerätebau, Netheler & Hinz GmbH, 2000 Hamburg 63 (Hummelsbüttel), Barkhausenweg 1) - for 20 microlitre quantities - or a glass micropipette (E-MIL) - for 50 microlitre quantities. Electrophoresis was then carried out exactly as described for chromaffin granule membrane samples (see previous

chapter) with 0.1%(w/v) SDS in 0.1M sodium phosphate buffer pH 6.5 as electrolyte. Once again, a small amount of bromophenol blue was included in each sample, to act as a "tracker dye". Electrophoresis was continued until the dye band had migrated to within ca. 1cm of the end of the gel (usually after 2½ hours).

Sectioning of Gels After Electrophoresis.

At the end of the electrophoretic separation the distance of migration of the tracker dye (from the origin) and the length of the gel were measured and the gel was removed from its tube by "rimming", in the usual way. The gel was placed in a small trough, made of thin brass sheeting, and frozen solid by surrounding the trough with crushed dry ice. The frozen gel was then transferred to the platform of a McIlwain-type sectioning apparatus (The Mickle Laboratory Engineering Company, Gomshall, Surrey). This platform had first been covered with a layer of adhesive tape, over which was placed a layer of filter-paper. Just before positioning the frozen gel on the filter-paper, the latter was dampened with distilled water. The gel cylinder was allowed to soften just sufficiently to make good contact with the filter-paper layer, before being aligned perpendicular to the sectioning-plane (parallel to the line of motion of the platform) and frozen to the platform by heaping powdered dry ice over it.

When the cylinder had adhered firmly to the moving platform, the end of the gel was exposed and allowed to thaw slightly (the thawing being hastened by blowing gently onto the exposed part of the

gel). The platform was then manually brought underneath the cutting-arm, which holds a razor-blade, and the slice-counter was set to zero. Sectioning was then begun, with the thickness control set to give sections of 1mm thickness, and each slice in turn was picked up with forceps and placed into a separate glass scintillation vial.

Treatment of the Sections.

When sectioning was complete, 1ml of Soluene 100 (Packard Instrument Ltd., Caversham, Berks) was added to each vial containing a slice of gel or aliquot of sample solution and the vials were tightly capped. After standing overnight at room temperature, the contents of each vial were mixed by touching the vial on a rotary mixer and the vials were placed in trays and heated in an oven for 6 hours at 60°. After cooling, the contents of each vial were once again mixed and the vials were again left overnight at room temperature before scintillation fluid was added. The scintillation fluid used was a solution of 0.5%(w/v) 2,5-diphenyloxazole (Scintillation grade PPO, Nuclear Enterprises Ltd., Sighthill, Edinburgh 11, Scotland) and 0.05%(w/v) p-Bis(2-(5-phenyloxazolyl))-benzene (scintillation grade POPOP, from the same suppliers) in ANALAR toluene (BDH Chemicals Ltd., Poole, Dorset). 10ml of this scintillator were added to each vial and the vials were allowed to stand overnight at room temperature before measuring the radioactivity in a Beckmann LS-200B liquid scintillation spectrometer.

Scintillation-Counting and Calculation of Results.

If the ganglion proteins had been labelled with tritium the "^{3}H" gate of the scintillation-counter was used. If the proteins were labelled with ^{14}C-leucine, the radioactivity was measured in both the "^{14}C" and the "^{14}C-WITH-^{3}H" gates. With an amplifier setting of 300, the upper energy limit of both of the latter two gates lies above the maximum energy for β-decay of ^{14}C, and the lower energy limit of the "^{14}C-WITH-^{3}H" gate lies above the maximum energy for β-decay of tritium. Any chemiluminescence in the samples (which is a problem often encountered with alkaline samples in scintillation fluid) appears to have an energy limit similar to that of tritium and is not recorded in the "^{14}C-WITH-^{3}H" gate. By contrast, the "^{14}C" gate is open down to much lower energies (as is the "^{3}H" gate), where such an effect might contribute to the number of "counts per minute" recorded.

Each sample vial was counted for 20 minutes in the "AUTO + STD" mode, in which an external standard reading is taken with every vial. Under these conditions, the counts in the "^{14}C" gate were recorded with a "2σ" accuracy of $\pm$ 3% or better and, in all but a few cases, the sample counts were always at least three times the background level, usually lying in the region of 200 to 5000 counts per minute (cpm).

The efficiency of detection was calculated from "quenching curves" constructed with ^{3}H- and ^{14}C-hexadecane standards, using

chloroform as a quenching agent. These curves related counting efficiency to the recorded "external standard" reading, and they were used to convert cpm to decays per minute (dpm). Counting efficiencies were usually in the region of 89%-92% for ^{14}C counted in the "14C" gate, 45%-60% for ^{14}C counted in the "14C-WITH-3H" gate, and 35%-45% for ^{3}H counted in the "3H" gate.

Data from the scintillation-counter (i.e.: the cpm in each channel and the external standard reading) were punched onto computer cards. A program was written in the Algol-60 programming-language which was used to convert this output to dpm and to plot the results in both histogram and smooth-curve form, normalised in terms of relative mobility (R_m x 100). This program was run on the University's central ICL 1906A computer and made use of the incremental graph-plotting facility available on this computer. For convenience in programming, the counting efficiency was taken to be linearly related to the external standard ratio over the range of values observed in these experiments. The program is set out in an Appendix to this thesis.

RESULTS

Investigations into the Reliability of the Analytical Procedure used to study Protein Synthesis in Ganglia.

The high resolving power of polyacrylamide gel electrophoresis makes it especially suitable for the analysis of protein synthesis in tissues, particularly if only small amounts of material are available. The superior cervical ganglion of the rat, a "typical adrenergic ganglion", has an average protein content of 60-80 μg and when it was decided to try to study the synthesis of proteins in this ganglion, therefore, the phosphate/SDS electrophoretic system, used to analyse chromaffin granule membrane proteins and described in detail in the previous chapter, was chosen as an analytical tool.

Because both ^{3}H and ^{14}C are radionuclides with rather low β-decay energies, and are thus most appropriately detected by liquid scintillation-counting, the radioactivities of the separated proteins cannot be measured in the presence of the gel matrix. Several approaches have been described towards the solution of this problem. Firstly, the gel matrix must be fractionated to give samples of a size that permits convenient processing without degrading the capacity of the electrophoresis system to discriminate between similar molecules and, secondly, the labelled proteins must be brought into contact with the bulk of the scintillation medium, so that the radioactive decays may be detected efficiently. Maizel (1966) has described an apparatus which disperses the gel by extruding it through a fine orifice, into

a stream of buffer which is split into fractions for separate analysis. A more commonly used technique (adopted in the present work) is that described by Loening (1967), in which the gel is frozen onto a movable platform and sliced into thin transverse sections by chopping with a mechanically-driven razor-blade.

Once the gel has been disintegrated or sliced, the protein may be extracted from the gel or the gel may be dissolved by a chemical method. Thus, hydrogen peroxide has been used to dissolve gels cross-linked with N,N'-methylene bisacrylamide (Young & Fulhorst, 1965), while 2% periodic acid dissolves gels cross-linked with N,N'-diallyltartarimide (Anker, 1970) and a variety of strong organic bases will dissolve gels cross-linked with ethylene diacrylate (Choules & Zimm, 1965; Peacock & Dingman, 1967; Weinberg & Penman, 1968). Paus (1971) found that dilute (2.5%) gels cross-linked with N,N'-methylene-bisacrylamide can be completely dissolved by prolonged incubation with the solubiliser Soluene-100 at elevated temperatures, but gels of higher acrylamide concentration remain undigested. Karlsson & Sjöstrand (1971a), in experiments on rapidly-transported proteins from retinal ganglion cells, reported quantitative recovery of applied radioactivity when slices of 7.5% gels were extracted with this solubiliser and their extraction procedure was followed in the present work.

In a multi-step analytical procedure there are many factors which significantly influence the experimental observations and which may critically affect the interpretation of the observations. It is

particularly important to take experimental problems into account when a variation in observed response (such as a change in the electrophoretic labelling-pattern) is to be related to some (non)physiological event (such as chronic denervation or electrical stimulation of the ganglion). In the experiments to be described in this chapter, for example, the supposed "physiological" change (which is in any case a change occurring in an *in vitro* preparation) is separated from the observed effect, in this case a change in the labelling-profile, by a sequence of operations comprising the solubilisation of the labelled proteins in SDS solution, the separation of the dissolved proteins by electrophoresis, sectioning of the acrylamide gel, chemical processing of the resulting slices, scintillation-counting of the solubilised radioactivity and computation of the final profile. Since neither Karlsson & Sjöstrand (1971a), whose extraction method was used in our work, nor Loening (1967), who described experiments in which a gel-cutter of the same manufacture was used, published any appraisal of the likely sources of error in their experiments, some emphasis was placed in my investigations on trying to identify and, if possible, eliminate variation arising from "technique".

Labelling and Extraction of Ganglion Proteins.

Under the incubation conditions used in labelling-experiments, the incorporation of radioactive L-leucine into a TCA-precipitable component of the ganglia was found to be linear with time, after the

first few minutes, for a period of at least 6 hours, and the rate of this incorporation was not affected by the presence of 250 I.U. of Penicillin G per millilitre of incubation medium (G. Toschi, personal communication). The solubilisation of this incorporated radioactivity was brought about by treatment with SDS, at a detergent-to-protein ratio of ca. 10:1, which was sufficient to solubilise all but a small amount of lipid or collagenous material of low specific radioactivity.

If the ganglia had contained any radioactive free leucine, not incorporated into protein and not washed out by the "chases" with non-radioactive leucine, this would have been present in the samples applied to the polyacrylamide gels for electrophoretic analysis. The electrophoresis was carried out at a pH (6.5) which is quite close to the isoelectric point of leucine (5.98), and if free leucine were present in the sample its migration in the gel might obscure the pattern due to the labelled proteins (if its relative mobility, which will be strongly dependent on pH and ionic strength, were low, but positive), or (if its relative mobility were negative) it might migrate cathodally or (if its relative mobility were large and positive) it might run off the end of the gel, causing a loss of radioactivity from the original sample which would lower the observed overall recovery (see a later section for a fuller discussion of the factors influencing the recovery). It was therefore desirable to try to estimate the proportion of the radioactivity in the sample solution that could be precipitated by TCA, (which was, presumably, the radioactivity

incorporated into protein) and the proportion which was not precipitable, which was most probably present as free leucine.

Paggi and Toschi had previously used a method for determining protein-bound radioactivity in ganglia which was based on that described by Karlsson and Sjöstrand (1971b). Following this method, aliquots of the sample are applied to glass-fibre discs, dried and treated sequentially with TCA and organic solvents and extracted by heating with Soluene before adding scintillation-fluid. Unfortunately, this method was found to give entirely unreliable results when applied to the analysis of electrophoresis samples, probably as a result of interference with the TCA-precipitation by the SDS in the sample, or due to losses during the subsequent solvent-extraction steps.

In an attempt to estimate the free leucine content by a simpler method, I carried out a more conventional TCA-precipitation, on a micro-scale. 100 microlitres of 12.5% TCA were pipetted into a small centrifuge tube (capacity 400 microlitres) and 10 microlitres of sample were pipetted on top of this, followed by a further 100 microlitres of 12.5% TCA. The tube was then capped and shaken vigorously and left to stand on ice for 20 minutes. Two 10 microlitre aliquots were then withdrawn and delivered into scintillation-counting vials and the tube was re-capped and centrifuged in a Beckman "Microfuge" for 10 minutes (16,000 g_{av}). Two 10 microlitre aliquots of the supernatant were then withdrawn and placed into scintillation-

Table 6.1.

The percentage of radioactivity in ganglion samples which was not sedimented by TCA-treatment and centrifugation.

Extract No.:	1	2	3	4	5
Analysis (i):	13.62%	11.40%	11.37%	10.52%	14.60%
(ii):	13.77%	11.52%	9.97%	10.64%	12.80%

counting vials. After the normal Soluene-extraction procedure, the samples were counted.

There are several objections to this methodology which imply that results obtained in this way are only a guide to the true content of radioactive leucine. Firstly, the TCA-treatment and centrifugation may not have been sufficiently rigorous to ensure that all of the radioactive protein was confined to the pellet. Secondly, some of the free leucine might have become trapped by the precipitate. Thirdly, the recovery of radioactivity was unknown, since (for technical reasons) the pellet could not be analysed, and the recovery was assumed to be 100%. Fourthly, the radioactivity of the supernatant was low (ca. 150 cpm) and therefore could not be determined with great precision (the "2σ" counting accuracy was $\pm$ 5% in the ^{14}C-WITH-^{3}H channel in which the measurements were made).

Taking these objections into account, the percentage of the dpm remaining in the supernatant (which is taken to be the percentage of free radioactive leucine in the sample) was found, from duplicate analyses of five different electrophoretic samples (Table 6.1), to average 12.02% (standard error 0.50%) of the total radioactivity. This did not vary very greatly between samples and, in the absence of a better method for analysing SDS-containing samples, has been assumed to indicate the upper limit for contamination by non-incorporated radioactive leucine.

Table 6.2.

Relative Mobilities and Staining Intensities of Ganglion Proteins in 6% Polyacrylamide Gels.

Mean R_m x 100	No.	Standard Deviation.	Standard Error.	Staining Intensity.
3.105	2	0.177	0.125	Faint
6.885	2	0.205	0.145	Faint
11.290	2	0.169	0.120	Very faint
13.410	2	0.396	0.280	Very faint
15.660	2	0.099	0.070	Faint/medium
17.070	4	0.329	0.165	Faint
19.115	4	0.326	0.163	Medium
20.746	3	0.496	0.286	Very faint
25.513	3	1.109	0.640	Very faint
28.480	3	1.148	0.663	Medium
32.887	3	0.214	0.124	Very faint
36.100	3	1.096	0.633	Medium
38.915	4	0.834	0.417	Very faint
44.608	4	0.596	0.298	Faint
46.690	4	0.506	0.253	Medium/strong
49.935	4	0.307	0.154	Faint
53.480	4	0.646	0.323	Faint
58.360	4	0.292	0.146	Very faint
61.515	4	0.519	0.259	Medium
66.240	2	0.651	0.460	Faint
69.170	4	1.114	0.557	Faint
74.375	4	0.444	0.222	Faint
78.167	3	0.936	0.540	Very faint
85.653	4	1.344	0.672	Very faint
93.295	4	0.766	0.383	Medium/strong
97.425	2	0.658	0.465	Faint
101.200	3	1.135	0.655	Medium

Electrophoretic Separation of Ganglion Proteins.

Experience with the use of the phosphate/SDS buffer system for the analysis of chromaffin granule membrane proteins suggested that electrophoretic separation on 6% polyacrylamide gels gave highly reproducible results, and this conclusion was supported by examination of the stained patterns obtained from SDS-extracts of rat ganglia. Both the relative mobilities and the staining intensities of the ganglion proteins showed little variation between experiments. Mean relative mobilities and visual staining intensities from experiments on four different ganglion-extracts are shown in Table 6.2 and the appearance of one such stained gel is shown in Plate 6.1. The standard errors of the mean relative mobility values compare reasonably well (range: 0.070 - 0.672) with the standard errors of the mean relative mobility values of chromaffin granule membrane proteins (range: 0.268 - 0.452, see Table 2.2), despite the smaller number of measurements for the ganglion proteins, and there is no indication from these figures that the migration of the ganglion proteins was inherently less reproducible than for chromaffin granule proteins.

The reproducibility of the migration distances in the stained gels was matched, as will be shown later, by similarly constant values for the positions of the peaks in the radioactivity profiles and the two sets of R_m measurements were in good agreement, although the relative prominences of the peaks in the latter case did not, in general, follow the visual staining intensities very closely.

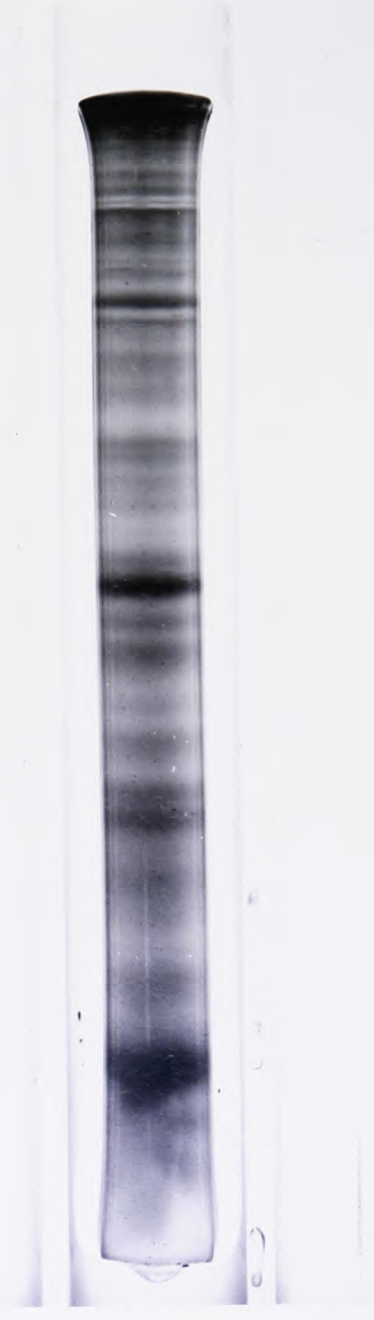


Plate 6.1. The Stained Band-pattern of SDS-solubilised Ganglion Proteins.

50 µl of ganglion-extract (containing ca. 40 µg of protein) were submitted to electrophoresis in 6% polyacrylamide gel, using the phosphate/SDS buffer system.

Because of the agreement between optical and radioactive detection methods, and the reproducibility of the relative mobility measurements derived from them, it was concluded that any variability during the electrophoretic separation did not have an important effect on the observed labelling-pattern and, in contrast to the procedure of Karlsson & Sjöstrand (1971a), the gels were not normally fixed and stained for visual examination before sectioning. Although the actual quantity of labelled protein in each sample was extremely small, the total amount of protein applied to each gel in a typical experiment was of the order of 20-50 microgrammes and it is likely that this was sufficient to act as a "carrier" for the radioactive proteins, ensuring regular electrophoretic migration.

Sectioning of Polyacrylamide Gels.

The McIlwain-type tissue-chopper used in our experiments for sectioning polyacrylamide gels was of the same type and manufacture as that employed by Loening (1967) in his studies of radioactive nucleic acids. The apparatus consists of a motor-driven chopping-arm, which holds a razor-blade and moves in a vertical plane, and a platform (to which the gel is frozen) which is advanced beneath the chopping-arm by the operation of a motor-driven screw thread. Although the pitch of the screw thread is constant, the distance of advancement of the table at each operation of the motor may be adjusted by setting a knob which governs the angle through which the screw is turned before each cutting-stroke. This knob has engraved markings permitting the

Table 6.3: Estimation of Sectioning Error in Gel-slicing.

Nominal Section Thickness (mm)	Mean dpm	Standard Deviation.	Standard Error.	Number of Slices.	Coefficient of Variation.
1.0	6527.0	298.9	70.4	18	4.58%
0.8	5136.7	253.0	113.1	5	4.93%
0.6	3833.7	519.5	232.3	5	13.54%
0.5	3201.1	236.3	89.2	7	7.38%
0.4	2645.5	127.6	42.5	9	4.82%

Nominal Section Thickness (mm):	1.0 : 0.8 : 0.6 : 0.5 : 0.4
Ratio of Mean dpm:	1.0 : 0.787 : 0.587 : 0.490 : 0.405

distance of advancement to be set at any value between 0.0 and 1.0mm, in steps of 0.05mm.

To determine the variation in slice-thickness when chopping 6% polyacrylamide gels with the apparatus, and to check the accuracy of the dial settings, a gel with an uniform concentration of radioactive label was prepared by including an arbitrary amount of ^{14}C-Acetyl coenzyme A in the polymerisation mixture before casting the gels. This uniformly-labelled gel was then frozen to the platform in the usual way and sections of 1.0, 0.8, 0.6, 0.5, and 0.4mm (indicated) thickness were cut and placed into numbered scintillation vials. After the sections had been processed and counted, the mean "dpm per slice" was calculated for each slice-thickness and the results are set out in Table 6.3.

This experiment showed that for three of the thickness settings (1.0, 0.8, and 0.4mm) the coefficient of variation (standard deviation expressed as a percentage of the mean) of the "dpm per slice" was of the order of 4.6 - 4.9%. The rather high value of 13.5% observed with 0.6mm slices was caused by an accident in sectioning in which the gel was not cut cleanly by the razor-blade, resulting in part of one slice remaining attached to the next slice. Because of the small number of slices of this thickness taken (5), this accident has had a disproportionate effect on the estimate of the sectioning error. By comparing the mean "dpm per slice", it was also possible to check the accuracy of the slice thickness settings (although the absolute values

of the section thicknesses could not be calculated from these measurements) and these were found to agree reasonably well (within ± 2.5%) with the manufacturer's calibrations.

The experimental factors which are not apparent from the figures given in Table 6.3, but which are nevertheless important, are the subjective ones of operational convenience and the frequency of "accidents" etc. All of the labelling-profiles illustrated in this chapter, with one exception, resulted from experiments in which the frozen gel was sectioned into 1mm slices. For a gel 6cm long, therefore, there were 60 slices to be treated separately throughout the rest of the analytical procedure. Although the sectioning accuracy does not appear to suffer when the thickness of the section is decreased, and it may therefore be argued that advantage should have been taken of the increase in longitudinal resolution that this would have afforded, it is clear that the number of samples will be increased to an unacceptable degree. The consequences of decreasing the section thickness may be summarised as being to increase the number of samples to be handled (and hence the length of time necessary for scintillation-counting and data-processing), to decrease the absolute amount of radioactivity in each section (and thus the accuracy of its detection by scintillation-counting) and to increase the length of time required for the sectioning of the gel. It will be shown later that a reduction of the sectioning thickness from 1.0mm to 0.6mm had little effect in improving the resolution of the proteins

in the ganglion-extracts.

Accidents in sectioning gels and the subsequent handling of the slices fall into three general categories: 1) uneven sectioning of the gel, 2) the gel coming unstuck from the platform during sectioning, and 3) the operator putting two successive slices of gel into the same scintillation-vial. Clearly, the third source of error is not inherent in the experimental procedure, even though it may be aggravated by its complexity, but both of the other accidents arise from problems associated with particular features of the method chosen for slicing the gels.

Adhesion between the frozen gel cylinder and the moving platform of the section-cutter is maintained by a thin layer of ice, and there are two points of experimental technique which are critical to the formation of this layer. Firstly, when the gel is placed on the moving platform, it must be allowed to thaw sufficiently for the whole length of the gel to make good contact with the layer of filter-paper. Secondly, care must be taken to avoid applying too much water to the surroundings of the gel, or the resulting layer of ice will be too thick, when it may impede the cutting of sections or crack, causing the gel to become unstuck from the platform. If the gel cylinder does come unstuck in this way, it is obviously extremely difficult to align the end of the gel accurately when re-freezing the gel to the platform, and the first section cut after re-freezing will not be exactly 1mm long, causing a spurious peak or trough to appear

in the labelling-profile. This accident most commonly occurs towards the end of the gel, when only a short length remains to be sectioned, and since the "dpm per slice" has usually fallen to a low value at this point in gels of ganglion proteins, this discontinuity in slice-thickness does not usually have a serious effect on the pattern observed.

Uneven section-cutting was not normally a significant source of error in gel-slicing at a slice thickness of 1mm, but was more frequent when thinner sections were cut. Successful sectioning is only possible over a rather narrow range of temperatures, since polyacrylamide gels of 6% acrylamide content have a rubbery consistency at room temperature and freeze hard when surrounded by dry ice. If the gel is too cold, the impact of the razor-blade when sectioning may cause the gel to shatter, or may result in the cutting edge of the blade becoming quickly blunted. If, however, the gel has been allowed to thaw too much, the gel tends to deform elastically, when uneven sections are obtained. During cutting, the thawing of the exposed end of the gel was hastened by blowing on it gently. Under these conditions, there will be a temperature gradient along the gel and the melted zone should extend just sufficiently far into the gel for that part of the gel directly under the razor-blade to have thawed to the optimal consistency for section-cutting. This situation is favoured by the adoption of a regular rhythm during sectioning.

There is one further aspect of experimental technique during sectioning which may result in a distortion of the labelling-pattern. It was found that it was not possible to heap a sufficient quantity of dry ice onto the gel for it to remain frozen throughout the entire period of sectioning, which normally took 45 - 60 minutes, and fresh dry ice had, therefore, to be added at intervals during this time. The addition of fresh dry ice causes the exposed end of the gel to freeze solid once again and the temperature gradient along the gel is upset. Since the gel may shrink slightly during this freezing and eventual re-thawing, the section thickness may vary slightly until the temperature gradient is re-established in the gel. When notes were made of the points at which extra dry ice was added during sectioning, it was found that this sometimes had the effect of introducing a small peak or trough into the profile, the effect extending over two or three slices on occasion. The increase or decrease in the "dpm per slice" attributable to this cause did not normally exceed 10% for the slice immediately following the addition of dry ice, and was much less for successive sections. Since this source of error was only recognised at a late stage during the investigations, the points at which extra dry ice was added are not, in general known.

Chemical Treatment of Gel Slices.

The chemical extraction procedure used in our experiments to leach out the radioactivity from gel slices into an homogeneous

liquid phase, where it can be detected by scintillation-counting, was the method of Karlsson & Sjöstrand (1971a), in which slices are treated with the tissue-solubiliser Soluene 100. Karlsson & Sjöstrand comment: "This procedure elutes all the radioactivity from the gel slices". They do not, however, present any experimental data to justify this claim, nor do they state what the overall recovery of radioactivity from the gels was, after electrophoresis, staining (omitted in our experiments), sectioning and chemical treatment.

The necessity for chemical extraction of the radioactivity was demonstrated in experiments with slices of the uniformly-labelled gel used to determine the accuracy of section-cutting (see above). A number of the 1.0mm slices were not treated chemically before adding the 10ml of scintillation-fluid and only ca. 350 cpm were recorded in these samples. After counting was completed, 1ml of Soluene was added to the contents of each vial and the vials were allowed to stand overnight at room temperature, before being counted for a second time. From the second set of cpm values, it was clear that only 5% of the radioactivity (^{14}C-Acetyl coenzyme A) was recorded if the chemical extraction step was omitted, suggesting that the β-particles emitted by the isotope were not sufficiently energetic to penetrate through the gel matrix, into the bulk of the scintillation-medium. (It was not necessary to heat the slices in the oven to achieve solubilisation of the radioactivity in this experiment, since Acetyl coenzyme A

is extremely alkali-labile and Soluene is a strong organic base).

In my experience the overall recoveries of radioactivity varied considerably between experiments separated from one another by time periods of several months, but remained almost constant within individual series of experiments. For example, in one group of analyses of four separate ganglion extracts, all prepared at the same time and all analysed in duplicate with the same batch of Soluene, the (abnormally low) overall recoveries of radioactivity were: 54.6%, 51.9%, 54.5%, 53.1%, 48.1%, 59.8%, 54.9%, and 57.0% (Mean: 54.25%, standard error of the mean: 1.22%). However, in an experiment on another extract of the same kind, prepared in an identical fashion at a later date and analysed with a different batch of Soluene, the overall recovery was 88.6%. There are a variety of factors which may affect the measured recoveries of radioactivity and, in view of the alarming discrepancy between apparently "identical" samples of ganglion-extracts, these factors must now be examined in detail.

The "overall recovery of radioactivity" is calculated as being the "dpm applied to the gel" (calculated from the volume of the sample and a duplicate measurement of its radioactivity) divided by the "total dpm recovered from the gel" (calculated as the summation of the individual dpm values, which have been corrected by the subtraction of a "blank" value for the background radiation and random thermal events counted by the scintillation-counter in the absence of radioactivity in the sample solution). The first of these

(dpm applied to the gel) is susceptible to fluctuation from errors in pipetting the small volumes of highly radioactive liquid sample, and from errors arising from the fact that different types of pipette were used to measure out aliquots of the sample for determination of its radioactivity (Drummond microcapillaries) and to apply a known volume of sample to the gel for electrophoresis (Eppendorf or glass constriction pipettes). The calibrations of these three different kinds of pipette were checked against one another by using them to pipette aliquots of the same (radioactive) solution (containing an arbitrary amount of ^{3}H-inulin, dissolved in 0.01M sodium phosphate buffer pH 6.5, containing 1% SDS and 10% sucrose) and quoted recoveries include a correction for the discrepancy in dispensed volume where necessary (only significant in those experiments in which a glass constriction-pipette was used to apply the sample to the gel). This same calibration experiment also indicated the variation to be expected in the dispensed volume. For the Eppendorf pipette the standard deviation was 0.87% of the mean, while the corresponding figures for the microcapillaries and constriction-pipette were 0.92% and 1.18% respectively. Clearly, this small degree of variation does not explain the large difference in recoveries reported above.

The "total dpm recovered from the gel" may be increased or decreased by errors arising during any of the four principal stages of the experimental procedure: electrophoresis, extraction of radioactivity from the gel slices, scintillation-counting and

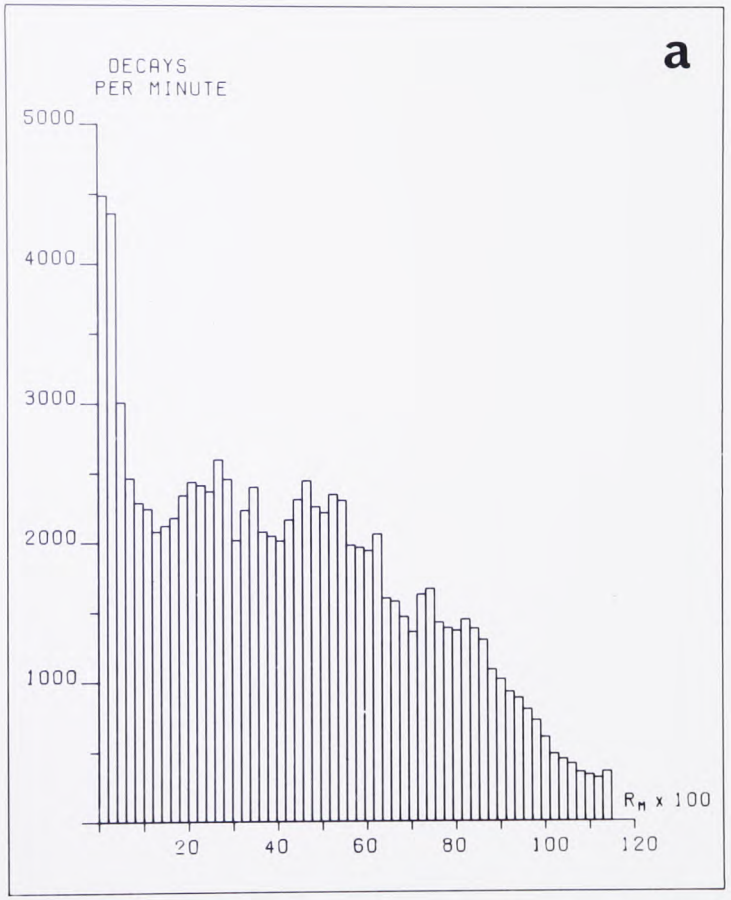
calculation of dpm. The scintillation-counting and data-processing steps in the analysis were found not to contribute significantly to the variation in recoveries and they are discussed in fuller detail under separate section-headings.

Loss of radioactive material during electrophoresis may occur either because the material migrates off the end of the gel in the direction of the anode (R_m x 100 greater than ca. 115) or because it never enters the gel, or migrates towards the cathode. It seems unlikely that radioactivity was lost for the first of these reasons, since even a small molecule such as a peptide would not be expected to migrate much faster than the dye, itself a small molecule, and the "dpm per slice" did not rise towards a maximum at the end of the gel (The increase in the final slice, seen in some profiles, is due to increased section thickness and is therefore artifactual). On the other hand, it is possible that some labelled material was lost because it did not enter the gel from the sample solution and a peak in the dpm-profile was often seen at the top of the gel (R_m x 100 less than 5); however a band of very low mobility (R_m x 100 = 3) was also noted in the staining pattern and the peak of radioactivity may correspond to this component, rather than to material which did not actually penetrate the gel. Losses of radioactivity from this cause would tend to decrease the calculated overall recovery and different sample preparations might contain different amounts of such "undetected material", but the variation in the sample content of the "undetected

material" would have to be quite large to account for the observed differences in recoveries in this way. If this "undetected material" does, in fact, exist, it could be a quantitatively important component in the sample, but it is hard to see how it might be isolated and identified.

Another cause of variable or unusually low recoveries could be varying efficiency of chemical extraction of the radioactivity from the gel slices, using Soluene. It has already been shown (see an earlier paragraph) that efficient extraction of radioactivity away from the gel matrix is important, since unextracted radioactivity will not be detected by the scintillation-counting system, and it is noteworthy that the low recoveries commented on earlier were all associated with experiments in which the same batch of Soluene was used.

The efficiency of Soluene-extraction was re-examined, in view of this observation, since if it were found that some components (for example, radioactivity from proteins of high molecular weight) were more efficiently extracted than others (for example, radioactivity from proteins of low molecular weight) by a particular batch of Soluene this would have serious implications for the interpretation of the observed labelling-profiles. To investigate this possibility, two gels were run, each loaded with 20 microlitres of the same ganglion-extract, and each gel was then sliced into 1mm pieces, as before. One set of gel slices was then submitted to the standard extraction procedure, with a 6-hour period in the 60°C oven. Before



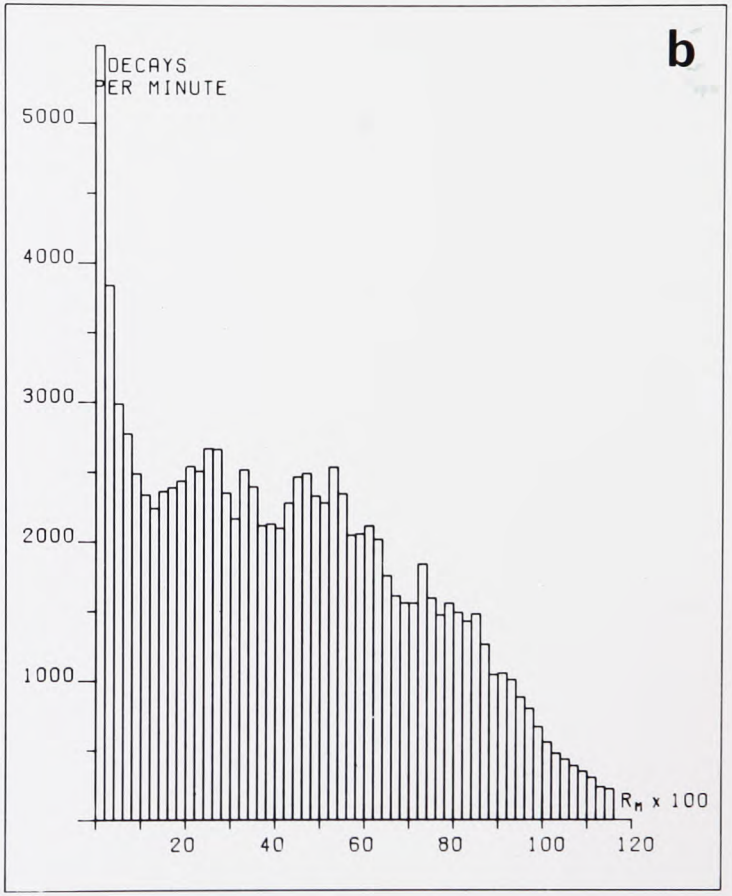


Fig. 6.1. Labelling-patterns Obtained after Different Soluene-extraction Times.
The gel slices were extracted at 60° for (a) 6 hours and (b) 12 hours. The same sample was applied to the gel in each case.

adding Soluene to the vials containing the other set of slices, 50 microlitres of distilled water were pipetted onto each gel slice to dampen it. The slices were then processed as normal, except that they were incubated in the oven for 12 hours instead of for 6 hours.

The recovery of radioactivity from the first gel (6-hour incubation, Fig. 6.1a) was 88.0%, while that from the second gel (12-hour incubation, Fig. 6.1b) was 92.6%. This difference (4.0%) was not materially greater than the differences observed when duplicate analyses were carried out by the standard method, with 6-hour incubations, and it could not be concluded, therefore, that prolonging the incubation time and dampening the sample (both recommended by the manufacturer as likely to improve extraction) had any significant effect on increasing the recovery.

The labelling-profiles from these two gels (Fig. 6.1a and b) were similar and resembled that of an earlier experiment, in which a sample prepared in the same way had given a recovery of only 59.8% (Fig. 6.2). A close examination of the pattern from this earlier experiment reveals the similarity with the patterns of Fig. 6.1a and b in two ways: firstly, the peak maxima occur at approximately the same $R_m \times 100$ values in all of the profiles and, secondly, the relative peak-heights in the earlier labelling-pattern are similar to those in the later experiments, although the amount of radioactivity in each slice is much smaller in the earlier experiment. As well as demonstrating the important point that there was no preferential extraction of radioactivity, a visual comparison of these three

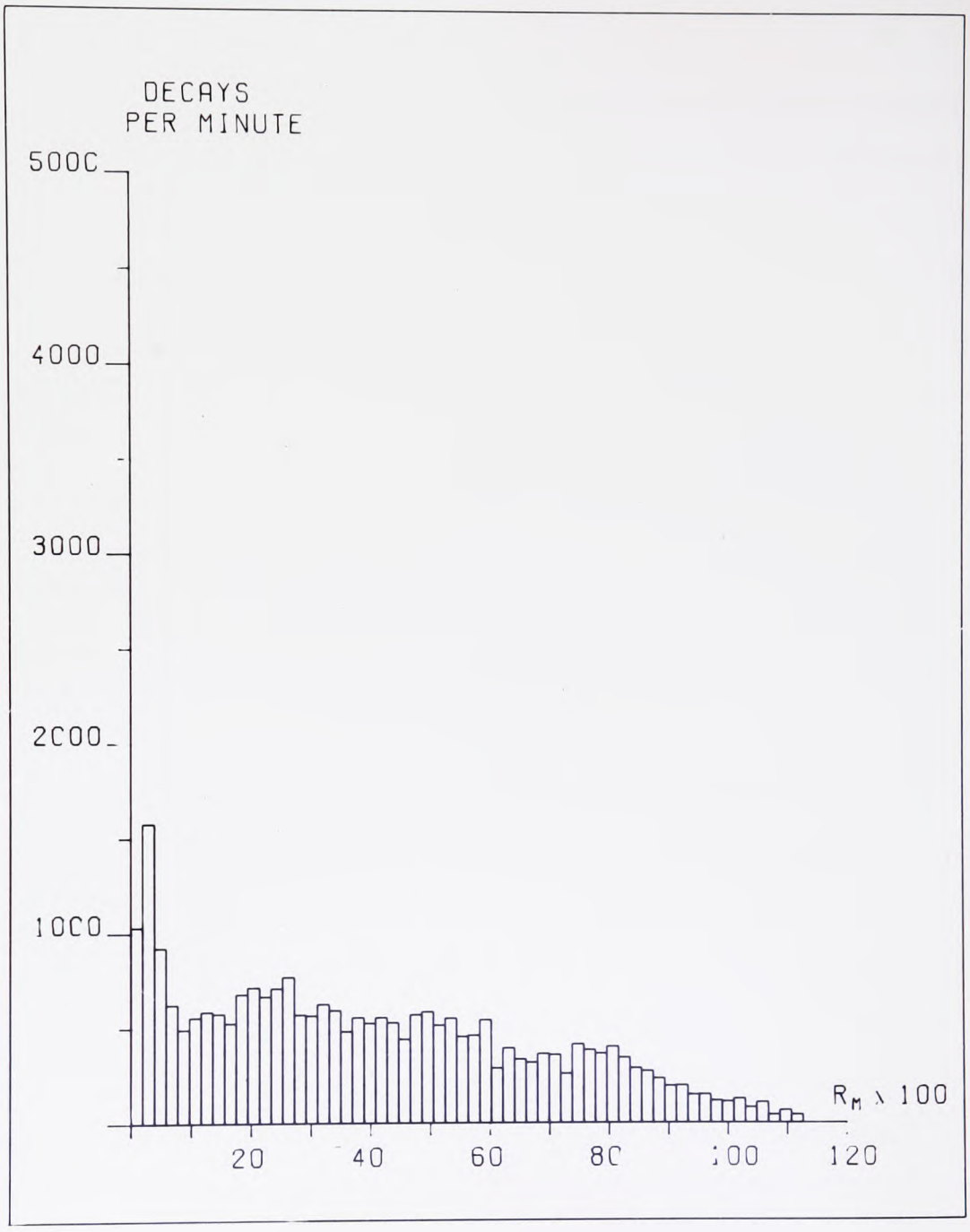


Fig. 6.2. The Labelling-pattern from an Experiment Showing an Anomalously Low Recovery of Radioactivity.

The sample used in this experiment was prepared in a similar way to the sample used in the experiments shown in Fig. 6.1.

profiles will serve to show the difficulties of making concise, semi-quantitative verbal comparisons.

Although the results do not clearly indicate whether losses in dpm, causing the observed low recoveries, stem from the loss of a variable proportion of "undetected material" during electrophoretic separation, or whether from inefficient extraction of radioactivity during chemical processing, they do show that these losses need not vitiate attempts to compare profiles from experiments with different overall recoveries.

Scintillation-counting.

The Soluene 100 tissue-solubiliser used in our experiments is a strong organic base, freely miscible with dioxane or toluene. Strongly alkaline samples may present problems in scintillation-counting, since a strong chemiluminescence may occur in the samples and the scintillation fluid may become discoloured (oxidised), causing quenching of the scintillation and hence lowered counting efficiency. Such problems are particularly acute in the scintillation-counting of ^{3}H in aqueous samples using dioxane-containing media and a toluene-based scintillation-fluid was preferred for the present work, for counting both ^{3}H- and (more usually) ^{14}C-containing samples. With this system, chemiluminescence decreased to an undetectable level within 24 hours of adding the scintillation-fluid and the recorded counts showed no long-term decrease on re-counting, even after several weeks storage at room temperature.

As noted under "Methods", calibration-graphs were constructed to relate the external standard ratio to counting efficiency for both ^{3}H and ^{14}C. This calibration was repeated at intervals and the resulting graphs were found to be practically invariant. Two graphs were constructed for ^{14}C, one for each of the "gate" settings on the scintillation-counter and these were used independently when working out the dpm from experimental data. The validity of the calibration-graphs was evident from the fact that the recoveries calculated from each set (14C- or 14C-WITH-3H) of data agreed to within 2.12% (mean of 13 experiments, standard deviation: 0.843) for the same gels, the calculated recovery being larger when data from the 14C gate were used. This result also shows that there was no significant interference from sample chemiluminescence, since this would augment the counts recorded in the 14C gate, leading to substantially higher recoveries than those calculated from data recorded in the 14C-WITH-3H gate.

Calculation of Profiles.

Because each ganglion-extract was normally analysed in duplicate, using two separate polyacrylamide gels, the scintillation-counting of the slices produced large quantities of data, from which dpm were calculated and plotted in the form of profiles of dpm per slice versus relative mobility. Typically, an extract from ganglia labelled with ^{14}C-leucine yielded 120 lines of scintillation-counter output, each showing the cpm in both the 14C and 14C-WITH-3H channels and the external standard ratio. For convenience of handling this large amount of data,

a computer program was written (see: Appendix), in the Algol 60 programming language, and run on the University's central ICL 1906A computer. This program reads in data from the scintillation-counter (after punching onto cards) and produces printed output showing the calculated dpm for each slice, together with certain other information such as the original data (to permit the card-punching to be checked) and the R_m x 100 value at the mid-point of each slice (for quick location of the mobility values of the peaks in the profile), and it also produces a file of information in machine-code, which is subsequently processed by the incremental graph-plotter, to give a plot of the calculated labelling-profile. (The profiles illustrated in this chapter are photographic reductions of graphical output from this program and have not been amended or re-drawn).

In the program a linear function is used when calculating the counting-efficiency, for converting cpm to dpm. The calibration curves of counting-efficiency versus external standard ratio were found to be rather flat curves in the case of both isotopes, but over the range of external standard ratios recorded this linear approximation would not be expected to introduce a significant error. After the cpm for each slice had been converted to dpm, the "background dpm" (equivalent to 40 - 60 dpm) were subtracted. (To obtain this background or blank value, two vials containing only Soluene were put through the whole heating and scintillation-counting procedure, for each set of gels). If this background value is an over-estimate of the true

blank, the calculated overall recovery will be too low, but even in the most extreme case, in which no "background dpm" were subtracted, the increase in the calculated recovery was only 5% and this is certainly an over-generous estimate of the likely error.

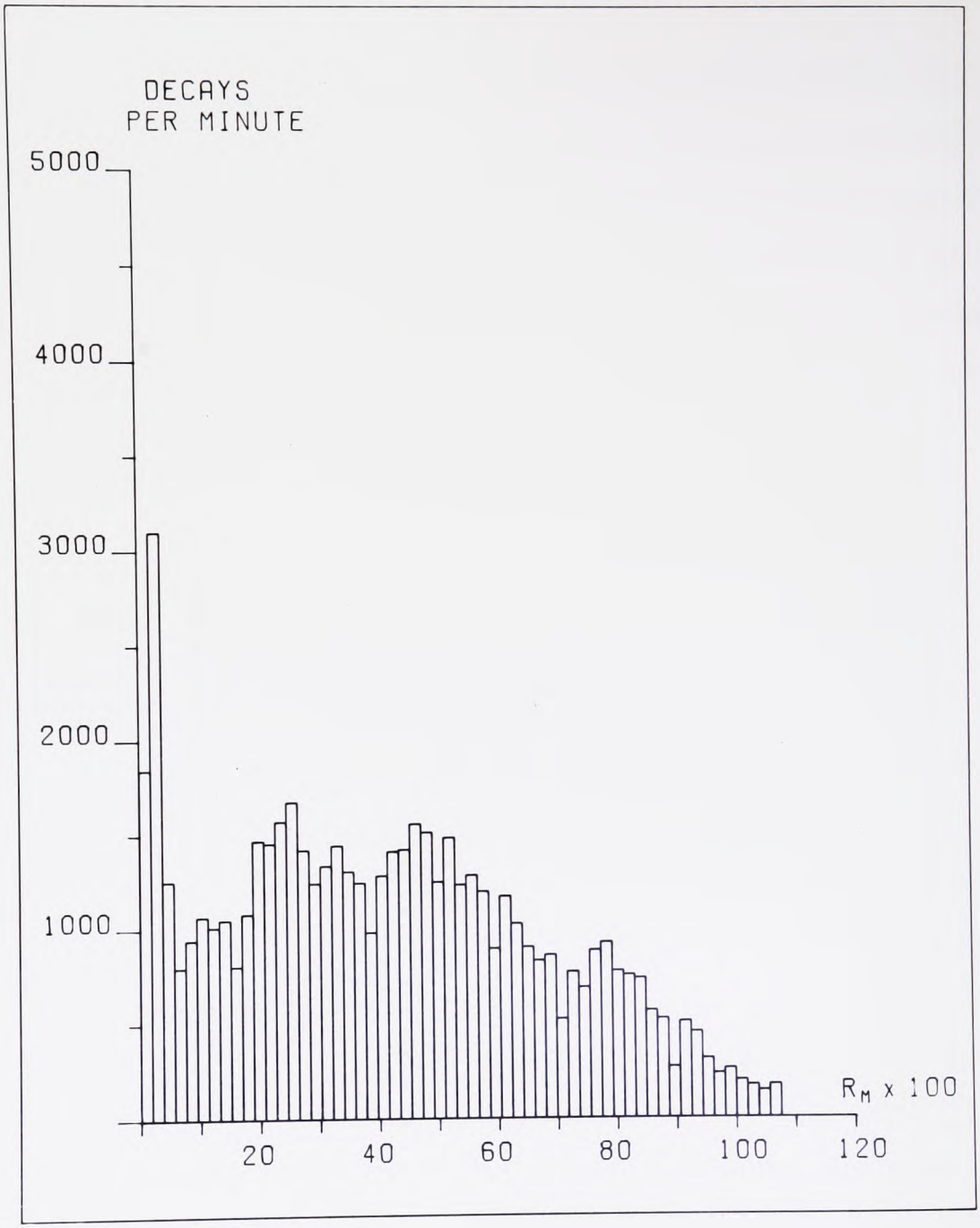


Fig. 6.3. The labelling-pattern [illegible] Cercalia Incubated for 7½ hours with ^{14}C-leucine.

Experimental Studies of Protein Synthesis in Rat Superior Cervical Ganglia: Labelling Profiles.

General Features of the Profiles.

It is clear that if the labelling-pattern is to be an useful and sensitive indicator of the physiological functioning of a ganglion, the exact shape of the profile must depend markedly on experimental variables such as the time of labelling, degree of stimulation and past history of the ganglion etc., however, it was found that, in nearly all cases examined, there are certain general features in common between the profiles. These features are exemplified by the pattern shown in Fig. 6.3, which is the profile obtained on labelling a resting ganglion for a period of 2½ hours, by incubation with ^{14}C-leucine. In all descriptions of profiles in this chapter the coordinates of peaks are given, for the sake of brevity and clarity, as numbers without explicit dimensions. The units implied are always those of the abscissa, that is "R_m x 100 units".

The first and most striking feature of the profile in Fig. 6.3 is that the radioactivity is not localised into a small number of extremely sharply-defined peaks, standing out above an unlabelled background, but is instead spread out over almost the whole length of the gel. Furthermore, ignoring the initial sharp peak at the origin (R_m x 100 less than 10) which is a variable feature, the peaks are not very pronounced, giving a "flat" appearance to the pattern. The envelope of the profile takes the form, after the first few

slices, of a humped arch whose shape may, perhaps fancifully, be likened to that of an archer's bow. After the first small peak at ca. 10, the envelope rises to a maximum at 25 which is the most prominent single feature of the profile and which is separated from the second major peak (at ca. 48) by a smaller hump in the region of 32-35. After the second major peak, the envelope describes an asymptotic decline towards the base-line which is interrupted by smaller, but still distinct, peaks at 55, 62, 68, 78 and 92. This characteristic pattern is most easily recognised at loadings giving 1000 - 2000 dpm per slice in the region 20 - 70, but the general features just outlined may also be distinguished in gels with higher loadings, in which the differences between peaks and troughs appear exaggerated by contrast, or lower loadings, in which the whole profile is much flattened.

Detailed Features of Profiles - Section-cutting, Slice-Thickness and Resolution of Overlapping Bands.

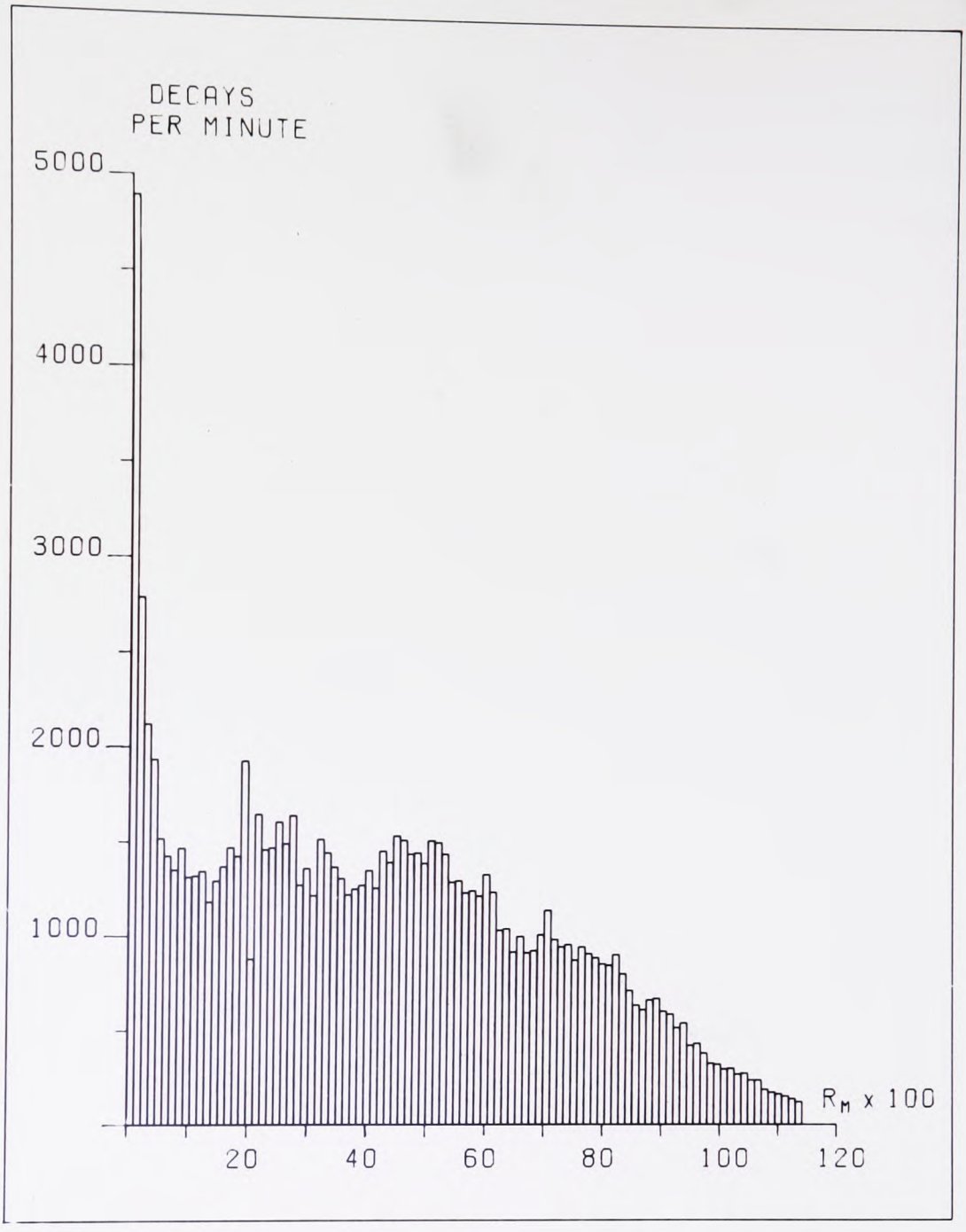
From the appearance of the profile shown in Fig. 6.3., and from others to be described later, it would seem likely that the observed pattern results from the summation of the radioactivities of many, closely-spaced, or overlapping, bands of labelled proteins. Staining with Amido Black demonstrated the presence of at least 27 components in gels of SDS-solubilised ganglia (Plate 6.1, see Table 6.2. for their relative mobilities and visual staining intensities) and it is probable that the greater sensitivity of the

radiochemical detection method reveals further components not present in sufficient quantities to be stained by the dye. This degree of heterogeneity in the composition of the sample adds special complications to the description and interpretation of the profiles and both experimental and theoretical studies were made of the likely consequences of this complexity.

(a) The Effect of Slice-Thickness on the Pattern.

In an idealised labelling-profile, obtained with "infinitely thin" sections whose thickness was negligible compared with the spread and separation of the protein bands, the labelled proteins might be expected to appear as a series of approximately Gaussian peaks, separated from one another by areas devoid of radioactivity. As the thickness of the section is increased, so the resolution of the peaks will progressively be degraded with the effect of "smoothing out" or "flattening" the profile until, when the section thickness is large compared with the spacing of the peaks, neighbouring peaks can no longer be distinguished from one another. The three variables peak separation, peak width and section-thickness are inter-related in their effect on the labelling-pattern and only the last of the three can be varied without altering the conditions of electrophoresis.

The choice of 1.0mm as a suitable thickness for sectioning was made on the grounds of manipulative convenience, however in the upper region of the gel (the first 1 - 2cm) the separation between


DECAYS
PER MINUTE
5000
4000
3000
2000
1000
R_M x 100
20
40
60
80
100
120

the stained bands was observed to approach this distance and, in one experiment, the slice-thickness was reduced to 0.6 mm in the hope of improving the resolution of the peaks in the labelling-pattern. The sample used in this experiment was an SDS-extract of ganglia which had been chronically denervated *in vivo* and stimulated with carbachol *in vitro* (see a later section of this chapter for a more exact description of the procedure) and was an aliquot of the extract used to obtain the patterns shown in Fig. 6.1.a and 6.1.b. (Figure 6.1.b is reproduced overleaf to facilitate comparison with the pattern from the present experiment and has been re-numbered as Fig. 6.5.)

As can be seen from Fig. 6.4., reduction of the section-thickness from 1.0mm to 0.6mm did not lead to a dramatic improvement in resolution, although there was some gain in detail, as, for example, in the region 40 - 68 where the three broad peaks seen in Fig. 6.5. are split into 6 or 7 narrower bands in Fig. 6.4. Unfortunately, the pattern in this figure is disturbed by an artifact in the neighbourhood of 20, caused by an accident during sectioning, however there is excellent agreement with the profile in Fig. 6.5., and a comparison of the calculated recoveries of dpm from the gels (107,485 dpm, = 92.41% of the total applied - for Fig. 6.4., against 107,684 dpm, = 92.58% of the total applied - for Fig. 6.5.) indicates the high degree of reproducibility between experiments that may be achieved under favourable circumstances. In both experiments, the gel slices were dampened with 50 microlitres of distilled water before adding Soluene

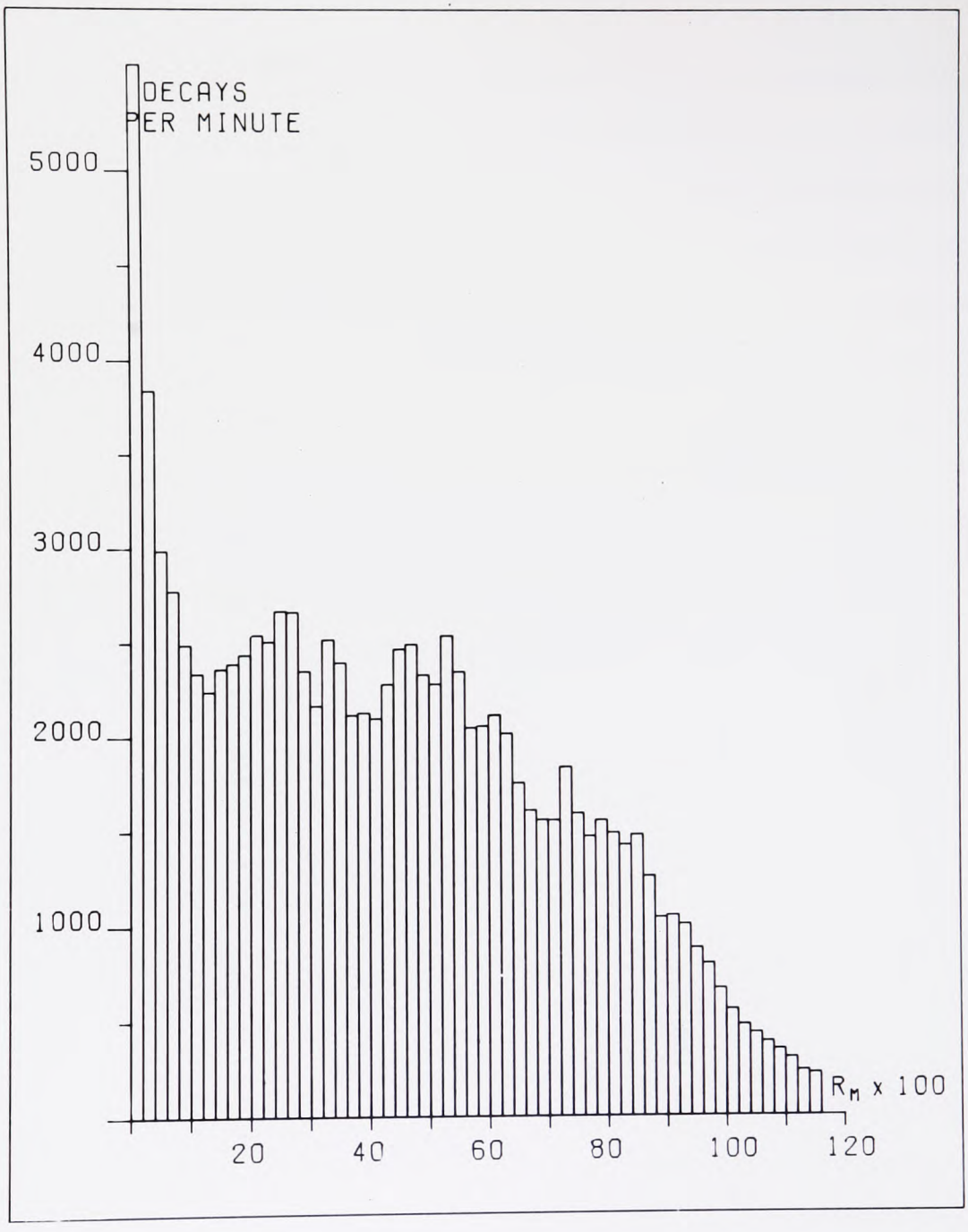
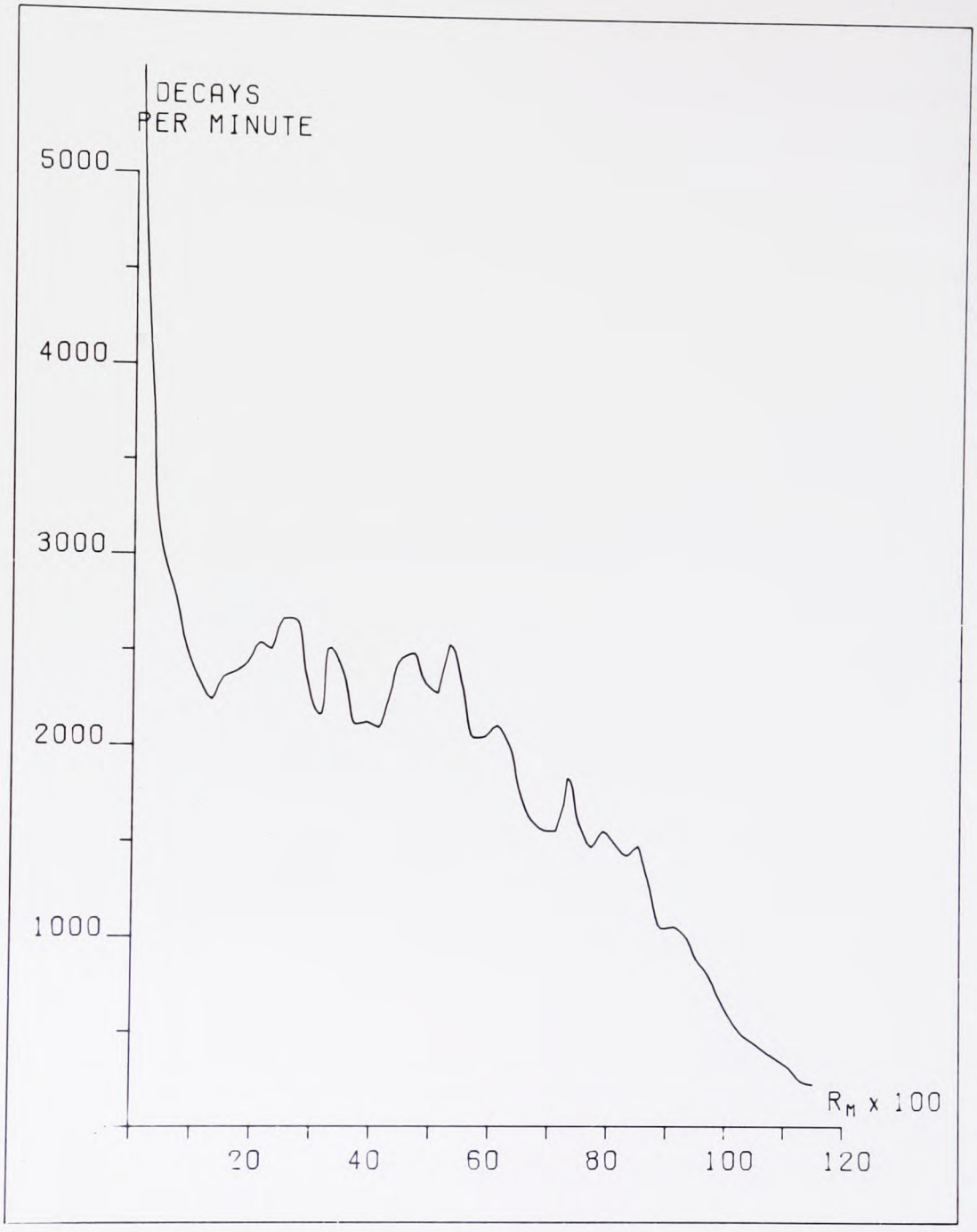


Fig. 6.5. The [illegible] pattern [illegible] Resolution.
This figure is identical with Fig. 6.4.b., and, like Fig. 6.4.,
[illegible] of a 12-hour toluene-extraction.

and the heating at 60° was continued for 12 hours. The last six slices in the profile in Fig. 6.4. are "synthetic"; that is to say that the actual cpm value for the last slice (which was considerably more than 0.6mm long) was re-distributed over six imaginary final slices in such a way that the total dpm was not affected, producing the usual falling profile right out to the (measured) end of the gel.

The results of gel-slicing experiments have always been shown in histogram form, with dpm per slice plotted against relative mobility (which is equivalent to slice number, since the sections were of uniform thickness), however, other authors (Loening, 1967; Gray & Steffensen, 1968; Karlsson & Sjöstrand, 1971a and c) have preferred to display their results as cpm or dpm per slice versus distance migrated, slice number or relative mobility using either smooth curves (drawn by eye?) to depict the labelling-pattern or joining the mid-points of the slices with straight lines to give the profile. In one case (Fig. 1A and 1B of Karlsson & Sjöstrand, 1971a) the slice numbers do not even fall at regular, linear intervals along the abscissa. The author believes that the histogram of dpm per slice versus relative mobility has advantages over other methods of plotting results, since it presents actual quantitative data (dpm) for radioactivity and the use of relative mobility units on the abscissa obviates problems of comparison betwen experiments caused by variation in migration distance: however, the computer program written for the calculation and plotting of results includes a


DECAYS
PER MINUTE
5000
4000
3000
2000
1000
R_M x 100
20
40
60
80
100
120

section of instructions which produce an alternative profile, in the form of a smooth continuous curve, through the mid-points of the histogram bars (slices), computed by a subroutine in the software library of the graphical output system (OXGHOST) of the 1906A which fits cubic curves through successive data points. (The routine is called CURVEO in the computer program given in the appendix). As an example of this kind of output, Figure 6.6. shows the results of the experiment with 1.0mm slices (Fig. 6.5.) plotted in smooth-curve form. This type of plot is very much influenced by large fluctuations in dpm between neighbouring slices (when the computed curve may "overshoot" in an unacceptable way) and, since the histogram output was found to be easier to interpret, it was not much used in the present studies.

(b) A Theoretical Examination of Gel-Slicing.

So far, differences between the patterns obtained from a single sample analysed in duplicate, using two gels run either in parallel or in separate experiments, have been held to result from anomalous electrophoretic migration or from errors in section-cutting. There is, however, one reason, not yet considered, why two identical gels, sliced with perfect precision into uniform segments, may nonetheless give different profiles, and this derives from a problem which is inherent in the method chosen for gel-chopping.

When the frozen gel is brought under the razor-blade of the sectioning-apparatus at the commencement of sectioning, the end of

the gel cylinder cannot be aligned sufficiently accurately with the blade to ensure that the first section cut is exactly 1.0mm thick. This is partly due to the fact that the end of the gel is covered with a layer of melting ice and partly to the fact that the end of the gel is usually convex, instead of being perfectly flat, but the consequence of the uncertainty in alignment is that the first slice may be of any length ("x" mm), up to a maximum of 1.0mm ($x \ll 1.0$). If the gel-chopping is absolutely uniform thereafter, the ends of the subsequent sections will fall at distances of 1.0 + x, 2.0 + x, 3.0 + x.....(n-1) + x mm from the origin of the gel. While this will have little significance, apart from altering the $R_m \times 100$ value of the peaks in the profile, so long as $x \cong 0.0$ or $x \cong 1.0$ and the slice-thickness (1.0mm) is much less than the peak widths and separations, it may have important consequences when these conditions do not hold, as is likely to be the case in the experimental studies described in this chapter.

As an illustration of the effects to be expected from such variability in the thickness of the first slice, let us consider a very much simplified example in which only two bands of radioactively-labelled protein are present in the gel, each being of "square-wave" shape, 0.3mm in thickness and each containing 15 dpm of radioactivity, the bands being situated at 3.15 and 4.55 mm from the (true) origin of the gel respectively. If we consider only those cases in which the actual thickness of the first slice (referred to as the "increment")

is a multiple of 0.1mm and calculate the "dpm per slice" to be expected in each of the first seven sections of the gel, we can see that six different patterns or "profiles" (of dpm per slice versus slice number) can be generated, even in this simple case with only two equally-sized protein peaks. The results of the calculations are set out in Table 6.4., and the six possible profiles will be designated (from the dpm in sections 4, 5 and 6) as being: 5-10-15, 10-5-15, 15-0-15, 15-5-10, 15-10-5, and 15-15-0.

Table 6.4.

Calculated "dpm per slice" for Model Gel, Containing Two Peaks of Radioactive Protein.

	Slice No.						
Increment (mm)	1	2	3	4	5	6	7
0.1	-	-	-	5	10	15	-
0.2	-	-	-	10	5	15	-
0.3	-	-	-	15	-	15	-
0.4	-	-	-	15	-	15	-
0.5	-	-	-	15	5	10	-
0.6	-	-	-	15	10	5	-
0.7	-	-	-	15	15	-	-
0.8	-	-	-	15	15	-	-
0.9	-	-	-	15	15	-	-
1.0	-	-	-	15	15	-	-

Although this example is clearly a highly artifical and over-simplified model of the real situation, it is nonetheless valuable as a guide to the problems which are likely to be encountered when interpreting the more complex, experimentally-produced patterns. Thus, in spite of the fact that in reality the protein bands are likely to approximate to Gaussian rather than "square-wave" peaks, the conclusion that different profile-shapes can arise from the same physical peaks, simply from variations in the exact position of sectioning, will still be valid. Furthermore, the model indicates how such variations in profile-shape are likely to confuse the interpretation of experimental histograms. For example, although it is obvious from the bimodal 10-5-15, 15-5-10 and 15-0-15 distributions that the pattern has been produced from two parent peaks, the profiles from the 5-10-15, 15-10-5 and 15-15-0 distributions are unimodal and could arise from a single component of either greater width or a (non-Gaussian) skewed shape.

As remarked earlier, the labelling-patterns from ganglia are extremely complex and probably result from the superposition of a large number of overlapping bands. It follows from this that the actual radioactivities of individual proteins cannot, in general, be deduced from an inspection of the experimental histograms; and that the patterns indicate only the "envelope" of the labelling, being a "sample" resulting from the representation of a continuous distribution (dpm versus distance) by successive values of a discrete variable

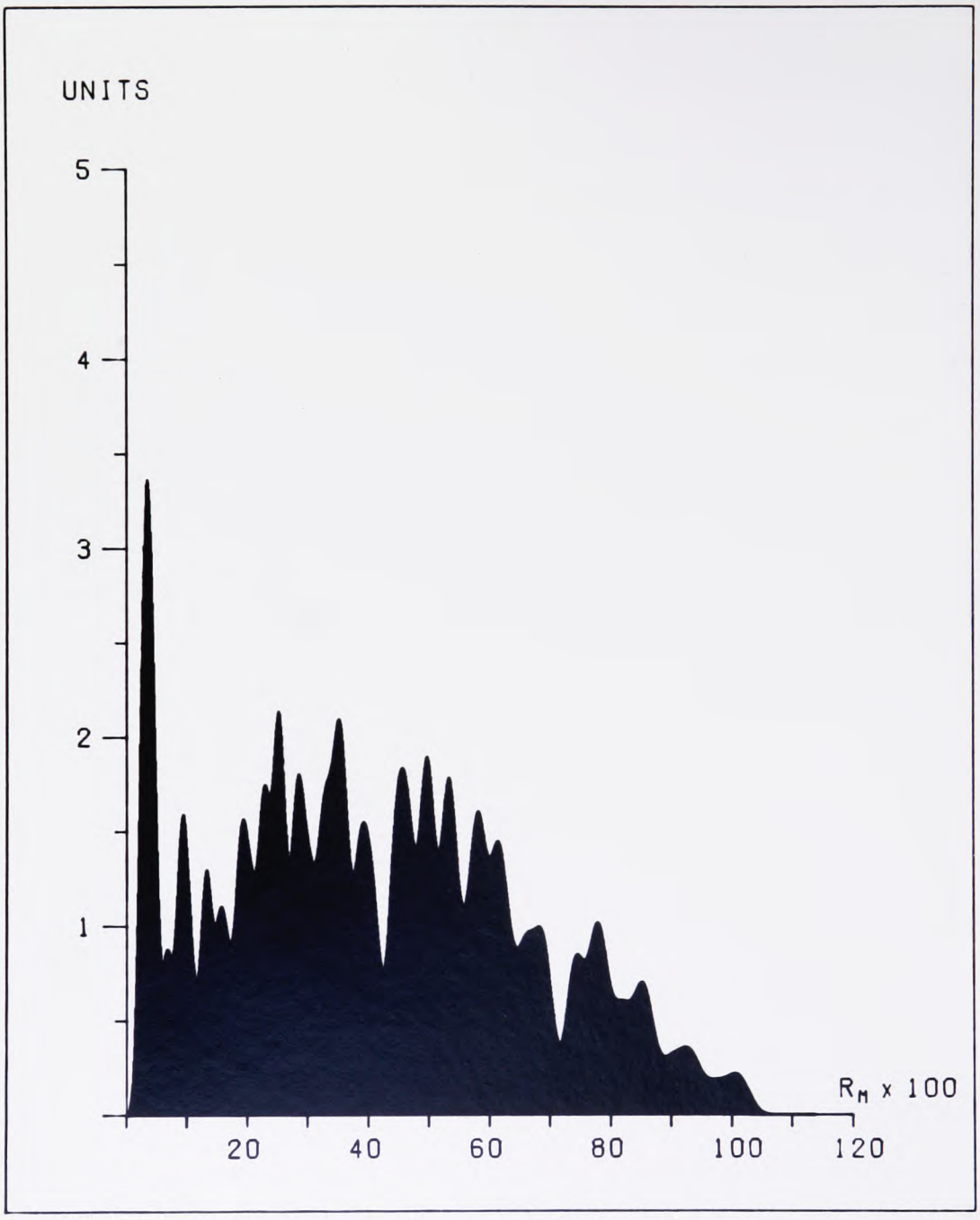
(dpm per slice). This being so, it can be seen that the actual effect of some experimental factor on protein synthesis within the ganglia may not be represented accurately in the histograms produced, since there is always an unknown and uncontrollable "sectioning error" which may distort the pattern.

In order to gain a better idea of how serious an effect this sectioning-distortion might have on the patterns produced by complex samples such as ganglion-extracts, it was decided to "simulate" the gel-chopping procedure mathematically, using a computer. The simulation was carried out in two stages, using two separate programs, and with the formidable number-handling capacity of a large digital computer available it was possible to make the mathematical model used for the analysis resemble the physical reality of the experiment more closely than in the simple example described above. The first program, called GAUSSCALC, was used to construct a quasi-continuous distribution of "radioactivity" versus relative mobility (R_m x 100) which was used to represent the "real" result of the experiment (i.e.: the true profile of the radioactivity, before section-cutting), while the second program, called CHOP, was used to "sample" this distribution, producing ten histograms at 0.1mm increments by a mathematical process designed to mimic the effect of mechanical slicing. Each of these programs was designed to produce graphical output in the same format as that from the program used to calculate the experimental histograms of dpm per slice versus relative mobility and therefore the units of

the abscissa in both GAUSSCALC and CHOP are R_m x 100, extending from 0 to 120. Since the true radioactivities of the individual components of the unsectioned profiles cannot be determined from the experimental histograms, the ordinates of these graphs are scaled in "units" (which have no physical significance) rather than in dpm, such that 1 "unit" in the theoretically-derived plots is notionally equivalent to 1000 dpm in the experimental patterns.

GAUSSCALC constructs a step-profile from any number of Gaussian curves, whose means and peak heights are read in as data when the program is run, by summing the heights of each of the individual peaks above the base-line, at points along the abscissa from 0 to 120 separated by 0.1 R_m x 100 units from one another. The program makes the assumption that the widths of the peaks increase linearly as their means increase, but since both the initial width (σ_o) and the factor governing the rate of increase (m) are also supplied as data when the program is run, their values may be chosen to produce whatever initial peak-width and rate of increase (if any) is desired. The height of the jth peak in the profile above the baseline at a point x is given by the expression (1), where $y_{max,j}$ is the height of the peak at its maximum (at its mid-point or "mean") and $MEAN_j$ is the position of the maximum of the jth peak on the abscissa.

$$y_{j,x} = y_{max,j} \cdot \exp \left(\frac{-(x - MEAN_j)^2}{(2(\sigma_o + m.MEAN_j))^2} \right) \quad \ldots\ldots\ldots\ldots (1)$$


UNITS
5
4
3
2
1
R_M x 100
20
40
60
80
100
120

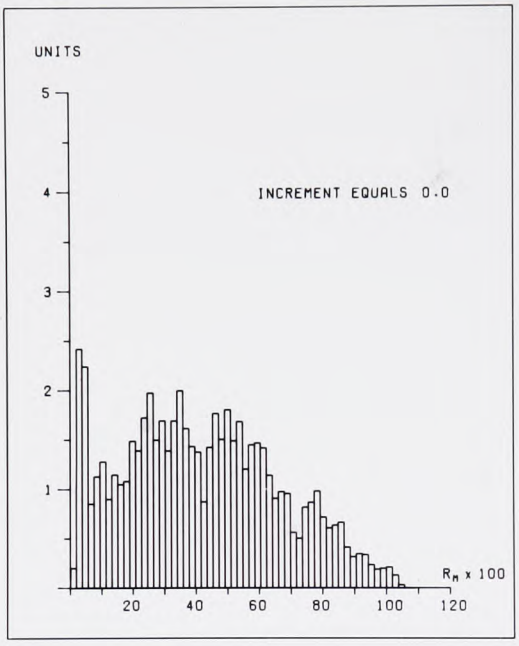
The height of the profile at each point, therefore, is obtained by adding together the $y_{j,x}$ values from each of the peaks and this height is stored as an element of an array of 1200 elements. When all of the elements have been calculated, the profile is displayed graphically and the successive array elements are written to an output file, to be used as data by the program CHOP.

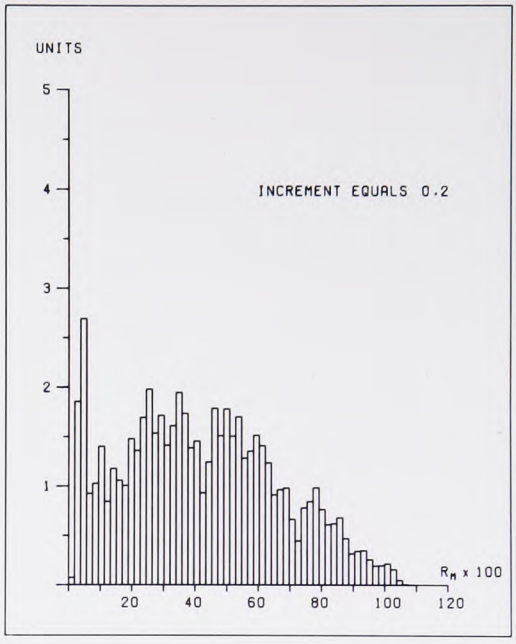
Figure 6.7 shows a profile calculated by GAUSSCALC in which the peak-width was allowed to vary from 0.8 R_m x 100 units at the origin to 1.8 units at R_m x 100 = 100, and in which 39 individual curves were used for the simulation. In the first attempt at creating the profile, 27 peaks of arbitrary heights were situated at points along the abscissa corresponding to the measured R_m x 100 values of the stained bands listed in Table 6.2. Their heights were then altered and extra components introduced on an empirical basis, until the histogram given by the program CHOP at an increment of 0.0 (corresponding to the situation where there is no sectioning-error) showed a resemblance to the experimental profile of Fig. 6.3. It must be emphasised that it was not intended to fit this experimental histogram exactly, since the aim of this computer simulation procedure was not to analyse the histogram to determine the positions and magnitudes of the radioactive peaks that gave rise to it, but rather to produce a profile which could generate a histogram of a complexity comparable to that observed in the experimental situation and which could be examined mathematically to discover the likely effects of sectioning error in a more realistic

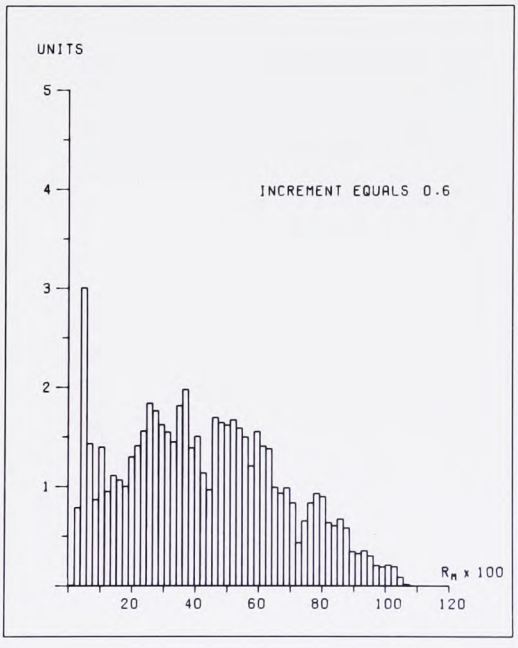
model system.

When a Gaussian-based profile has been produced in this way and output to a file, the file is "edited" to add on to the end of it the number of slices and the dye migration distance to be used in the "chopping" process when calculating histograms. This information is then input to the second program CHOP, for the calculation and plotting of the theoretical "labelling-profiles". CHOP performs these operations by firstly calculating the upper and lower bounds (in R_m x 1000 units) of each of the slices and then averaging the peak heights at each of the sample points in the Gaussian profile which fall within the limits of these bounds. The (very simple) programming methods used to achieve this need not be commented on, but mention must be made of one detail of the program, in order that the interpretation of the graphs it produces may be clear. In this program and in the graphical output it generates, the term "increment" is used to indicate not the thickness (x) of the first slice of the gel, as in the earlier discussion of the simple theoretical model, but, instead, the distance (1-x), which is the thickness of gel missing from the first slice. Thus, "increment = 0.0" corresponds to the situation in which the first slice is exactly the full 1.0mm in length, while "increment = 0.3" indicates that the first slice was 0.7mm thick, and so on.

When the profile shown in Figure 6.7 was "sampled" by CHOP, the resulting histograms confirmed the predictions of the simpler model about the changes in peak-shape that would result from variations in







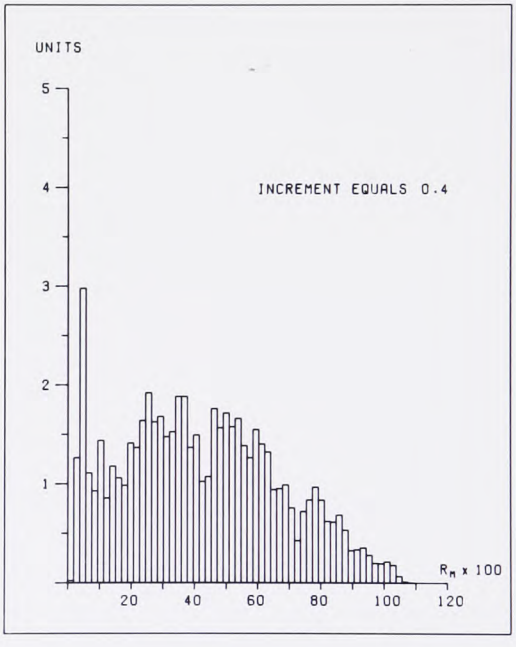


Fig. 6.8. Examples of Output from the Program CHOP.

These profiles, taken from the series of ten calculated by the program CHOP, are derived from the labelling-profile illustrated in Fig. 6.7., and show the patterns produced at increments of 0.0, 0.2, 0.4 and 0.6.

sectioning-position. In addition, they indicated how far the patterns from two experiments could differ from one another, as a consequence of sectioning-distortion, before it was necessary to conclude that they were derived from different underlying profiles. Four of the ten histograms produced from the profile of Fig. 6.7 are shown in Fig. 6.8 (the patterns at increments of 0.0, 0.2, 0.4 and 0.6mm) and these demonstrate clearly that variations in sectioning have too great a perturbing effect on the shape of the labelling-pattern for it to be worthwhile to attempt an interpretation of the "fine structure" of the experimental histograms. The effects on peak shape may be seen throughout most of the length of the gel, but are particularly noticeable at 35 and 40 and in the region 45 - 55 and at 58 - 60. Increasing the heights of the peaks in the original profile will cause these changes in shape to become even more pronounced, and this leads to the observation that, in the experimental situation, profiles from gels with high loadings of radioactivity are more difficult to compare and interpret than those produced at lower loadings.

Experiments with ^{3}H-leucine Labelling.

For most purposes, ganglion proteins were labelled by incorporation of ^{14}C-leucine, however the specific activity of commercially-available ^{14}C-leucine (330 mC_i/mmol) was not sufficiently high to give adequate labelling (and, hence, reliable profiles) at rather short ("pulse") labelling-times. Tritiated leucine is obtainable with much higher

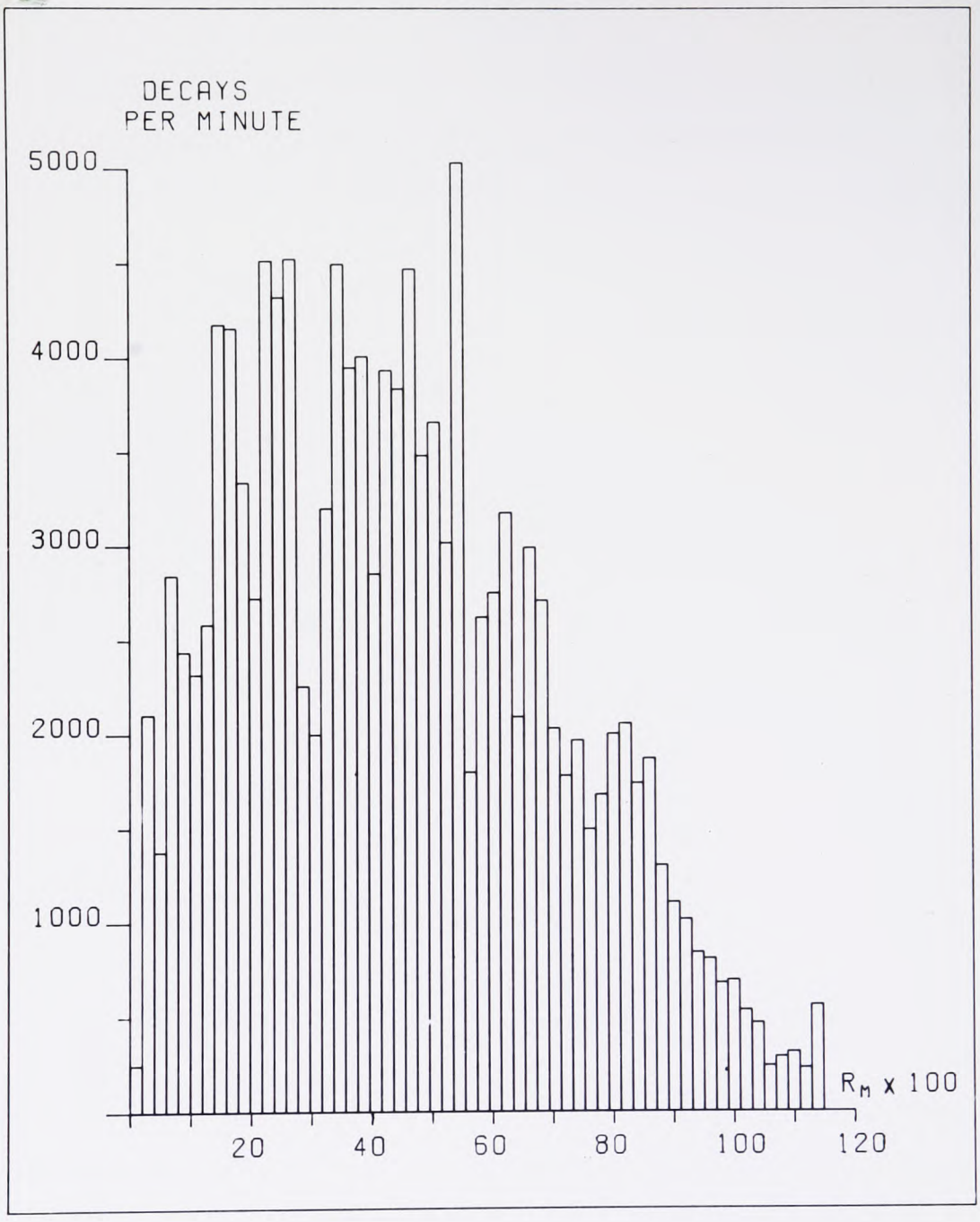


Fig. 6.9. The Labelling-pattern from Normal, Resting Ganglia Incubated for 2.5 hours with ^{3}H-leucine.

The ganglia were incubated with 5 mC_i = 20 nanomoles of ^{3}H-leucine (specific activity = 250 mC_i/mmol).

specific activity (L-leucine-4,5-^{3}H, greater than 30,000 mC$_{i}$/mmol) and was used for a small number of experiments for which this higher specific activity was desirable.

Figure 6.9 shows the profile obtained in an experiment in which normal, resting ganglia were incubated for 2½ hours with ^{3}H-leucine. The experimental conditions were the same as those used to obtain the ^{14}C-labelling pattern shown previously in Fig. 6.3, and the shapes of these two profiles appear similar to one another, when allowance is made for the increased dpm per slice in the ^{3}H-labelling pattern. The only marked discrepancy in relative peak-heights is at 55, where a large peak appears in the ^{3}H-labelling profile which is not present in the ^{14}C-pattern. Since this peak was not observed in another, earlier experiment (see Fig. 6.10 for the profile) in which a 2½-hour incubation with ^{3}H-leucine was carried out, it is probable that this peak is an artifact of sectioning, perhaps the consequence of adding more dry ice.

It is not unexpected that the labelling-profile from ganglia incubated for 2½ hours with ^{3}H-leucine resembles the profile obtained when similar ganglia were incubated for the same length of time with ^{14}C-leucine; indeed, it provides a test of the reproducibility of the experimental conditions, since, if the specific activity of the leucine is the same in both cases, substitution of a ^{3}H label for a ^{14}C label should be without effect on protein synthesis. The results given above, therefore, demonstrate that the extra problems introduced

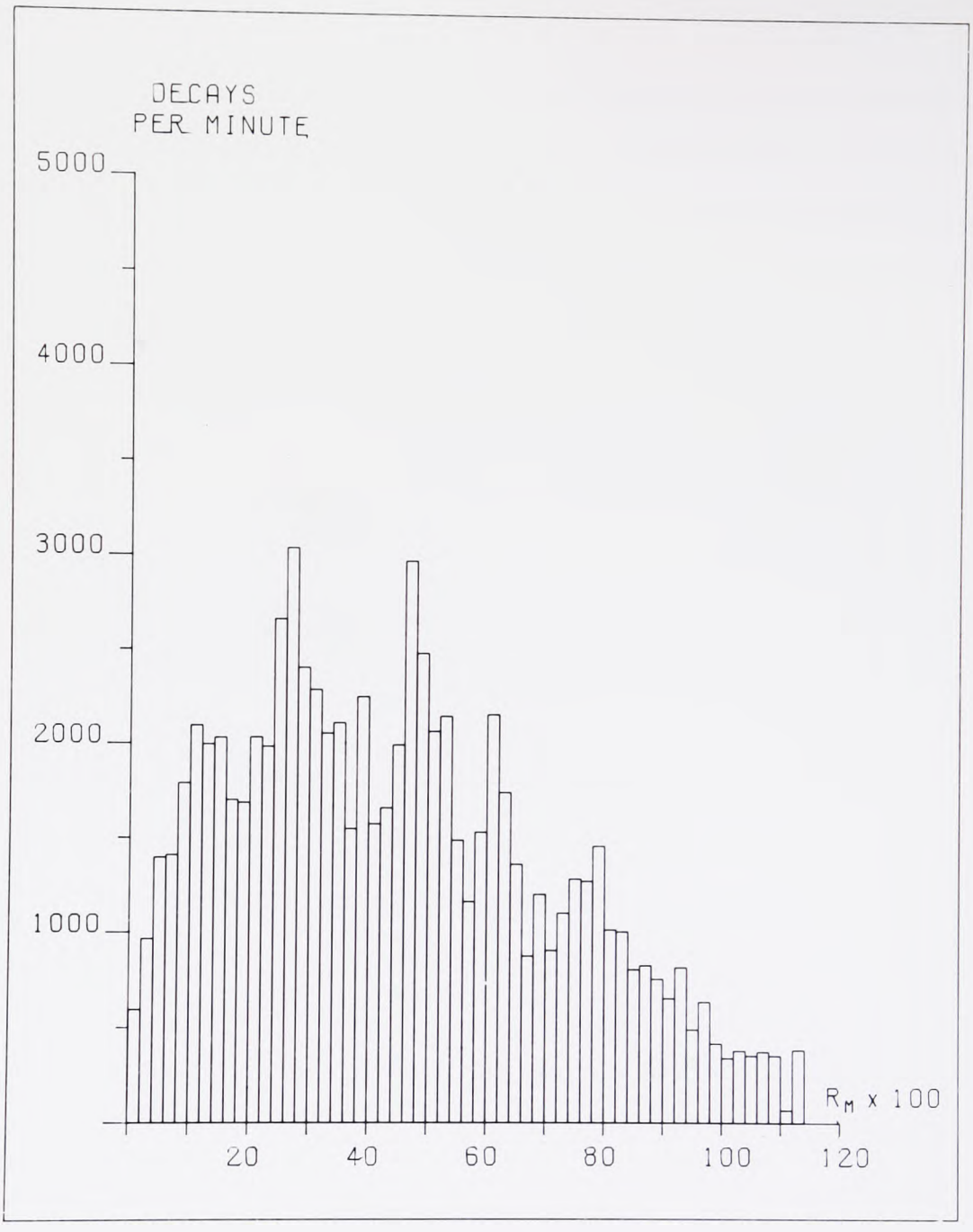


Fig. 6.10. An earlier labelling pattern obtained [illegible], showing results by incubation with ^{14}C-leucine.
The labelling and extraction conditions in this earlier experiment were identical with those given in the legend to Fig. 6.9.

by working with the very weak β-emitter tritium do not noticeably degrade the quality of the labelling-pattern.

The Effect of Shortening the Time of Labelling.

From the patterns arising from 2½-hour labelling with either ^{3}H- or ^{14}C-leucine, it was apparent that the peaks of radioactivity were compounded of many overlapping bands, representing a large number of newly-synthesised proteins. This may indicate that label was being incorporated into a large variety of cellular proteins other than those of immediate interest (those proteins with a role to play in the synthesis, storage, transport and release of neurotransmitter) during the relatively long period of labelling, and it was hoped that this interference from a back-ground of "general labelling" might be reduced by carrying out the incubation for a shorter period, using ^{3}H-leucine of a higher specific activity to compensate for the smaller incorporation.

The profile shown in Fig. 6.11 was the result of such an experiment, in which the labelling-period was of 20 minutes duration. Once again, the profile is dominated by two major peaks, in the regions 24 - 28 and 44 - 52, with an intervening hump at 32 - 37. The heights of the peaks in this profile appear to stand in the same relationship to each other as was noted with a 2½-hour labelling-period, and this profile is similar in shape to the pattern shown earlier (see Fig. 6.3) of 2½-hour labelling with ^{14}C-leucine, although the major peaks appear to stand out a little more from the background in this (20 minute)

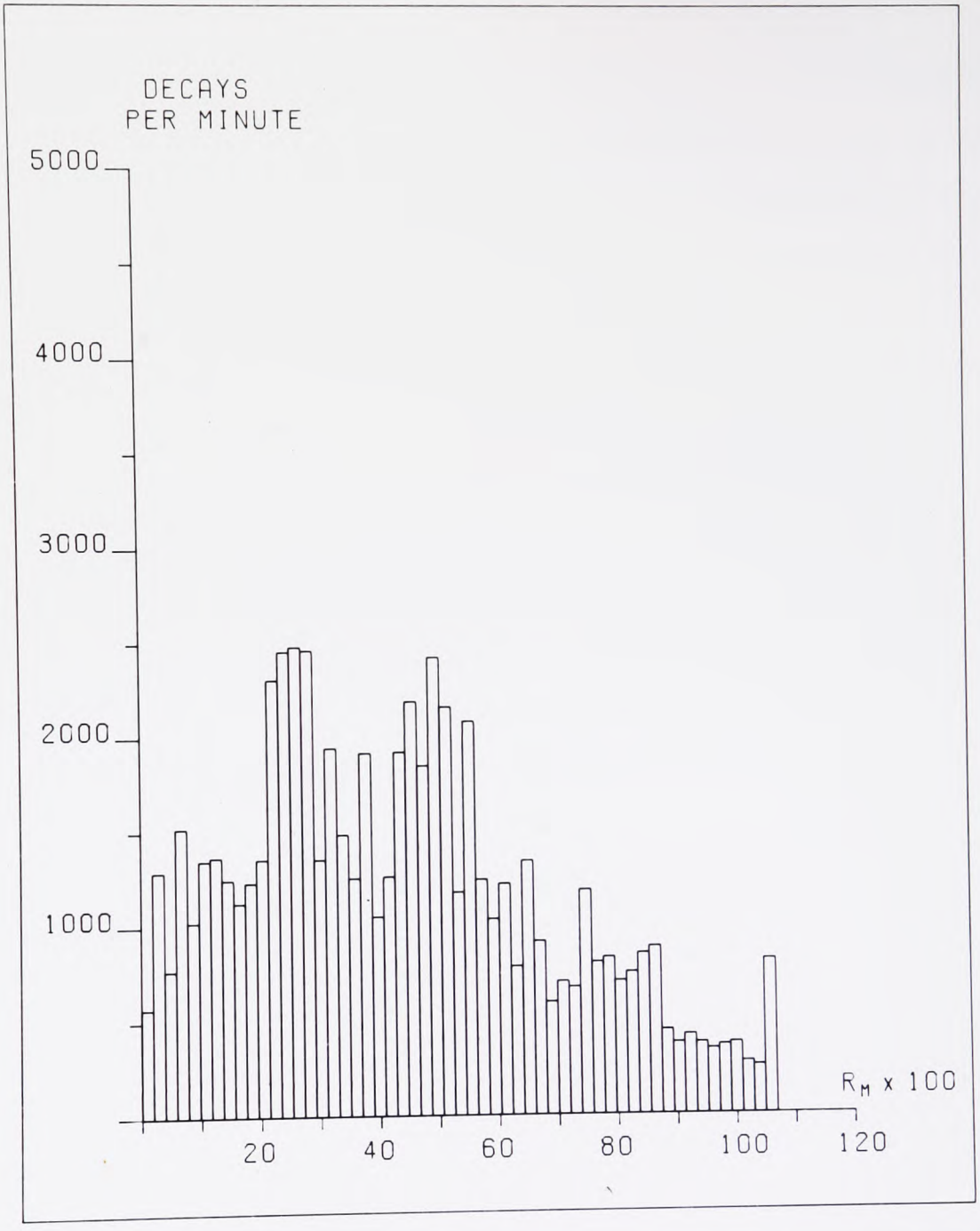


Fig. 6.11. The labelling-pattern from Formol-Heating Conclia Incubated for 20 minutes with ^{3}H-leucine.

For this experiment, [illegible] were incubated with $25\mu C_i$ = 25 nanomoles of ^{3}H-leucine ([illegible]-leucine, specific activity 1 C_i/mmole).

experiment.

As at the longer time, the profile is continuous and appears to result from the summation of the radioactivities of many overlapping bands. Although 20 minutes may, therefore, still be too extended a period of labelling for the back-ground of non-specific (general) synthesis to be much reduced in importance in the final profile, the technical difficulties of measuring small amounts of ^{3}H in gel slices are such that it would be extremely difficult to obtain satisfactory patterns with labelling-periods of 5 minutes or less. The similarity between the 20-minute and 2½-hour profiles was sufficiently great for it not to seem worthwhile adopting the shorter labelling-time as a standard in subsequent experiments, particularly in view of the greater convenience of using ^{14}C-leucine.

The Influence of Physiological State on Labelling-Experiments with Stimulated and Denervated Ganglia.

Although ganglion cells, in common with others, must manufacture a large range of proteins in order to maintain their biochemical and morphological integrity, they must be specially adapted to produce those proteins which are incorporated as functional (and possibly structural?) components of the neurosecretory vesicles continually exported from perikaryon to terminal, where they participate in the cycle of exocytosis. It is reasonable to suppose that the synthesis of this latter class of proteins, which for convenience may be termed

"neurovesicular" proteins, will be more responsive to changes in the environment of the ganglion, resulting from such "nervous" events as electrical stimulation of incoming fibres, or their degeneration after pre-ganglionic denervation, than those proteins concerned in the general metabolic and "housekeeping" processes of the cell. Since the labelling-pattern of the intact, resting ganglion proved to be extremely complex, experiments of this kind were performed, with the hope that changes in the labelling-patterns might indicate specific proteins likely to be associated with the neurosecretory vesicles.

Several experiments had been performed in which ganglia were incubated with radioactive leucine of the same specific activity as that used in the present studies, for the same length of time (2½ hours), however, an extra "control" set of ganglia (normal, resting ganglia) were analysed in this series of experiments, since the pre-incubation period had been increased to 4 hours instead of the more normal 30 minutes. This extended pre-incubation had been adopted because it was intended to compare patterns from the control (resting) ganglia with patterns from ganglia which had been subjected to supramaximal electrical stimulation of their incoming pre-ganglionic nerve fibres. McBride & Klingman (1972) have shown that large changes may occur, shortly after the commencement of the pre-incubation, in the intraganglionic content of free leucine in both resting and stimulated ganglia (although the alterations in level of free leucine do not follow the same patterns in both cases) but that these changes do not

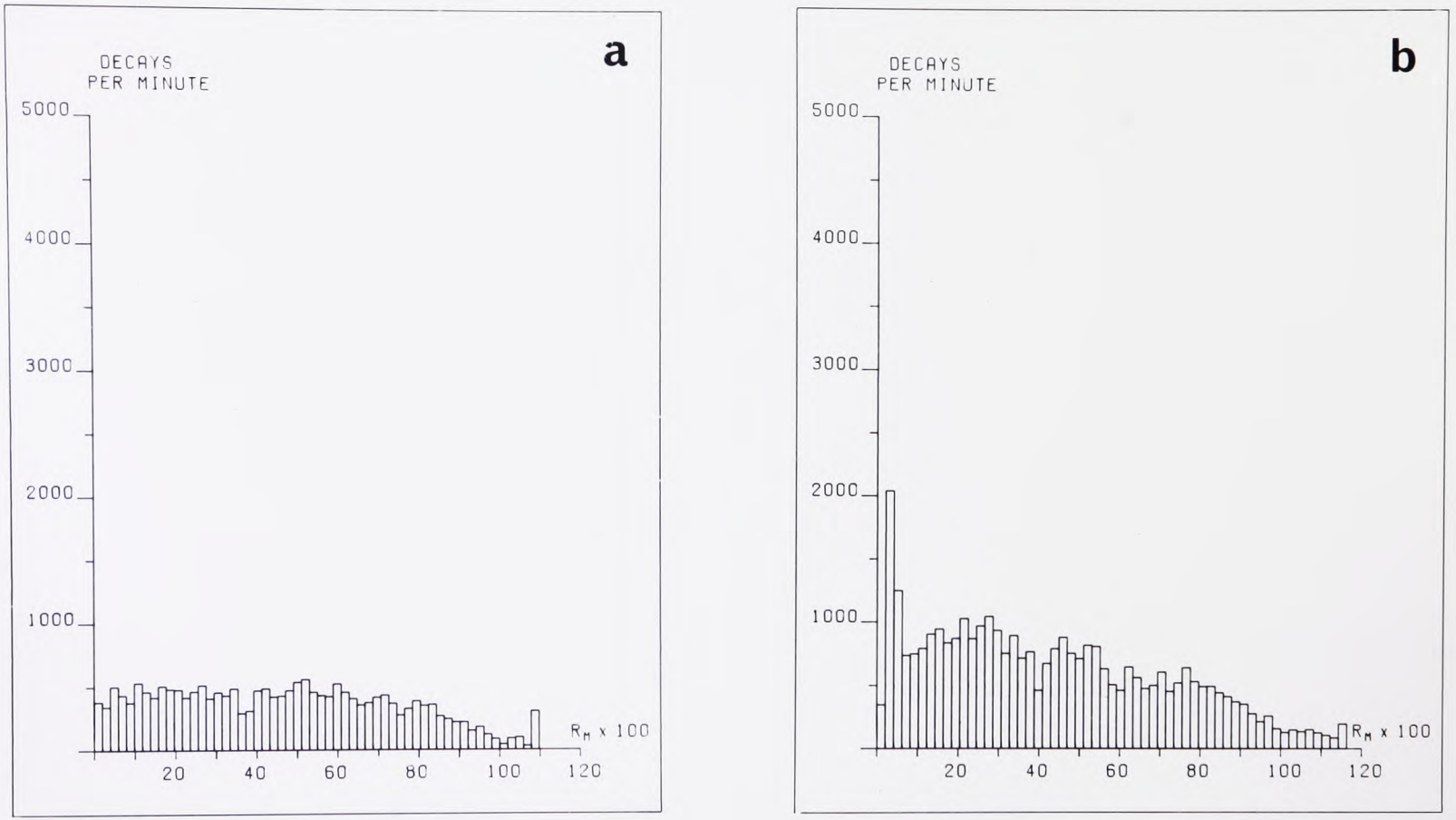


Fig. 6.12. ^{14}C-leucine Labelling-patterns from resting and Electrically-Stimulated Normal Ganglia. The ganglia were labelled for 1.5 hours, after a pre-incubation period of 4 hours. (a). Resting ganglia. (b) Ganglia stimulated at 10 Hz throughout the period of pre-incubation.

persist over a period of several hours.

When the pattern from these control, resting ganglia was examined (see Fig. 6.12.a), the labelling-profile was found to be extremely flat and low. In this experiment, and in the other three which follow, a new batch of Soluene was used which gave consistently low recoveries of radioactivity from the gels. Reasons were given earlier for thinking that this lower recovery did not complicate interpretation of the patterns, except by reducing the entire profile and thus making peaks much less obvious. The peaks in this profile (recovery of radioactivity 53.1%) fall at the same relative mobility values as in earlier profiles, even though they are much less pronounced than in (say) Fig. 6.3.

In a second experiment, the pre-ganglionic nerve-fibres of the ganglia were electrically stimulated throughout the 4-hour pre-incubation period, prior to addition of radioactive leucine. The resulting profile (Fig. 6.12.b, recovery of radioactivity: 54.6%) was more sharply-defined than that of the unstimulated control and once again closely resembled the patterns of earlier experiments, suggesting that under the conditions employed there was no enhancement in the rate of synthesis of one particular protein compared with the others, although activity in the region 10 - 20 was slightly higher than normal, as judged from the relative peak-heights. If the comparison to the control is made without consideration of the other profiles, observed on previous occasions, it would appear that the activity was slightly enhanced over the whole area 10 - 35, where the control profile is particularly flat.

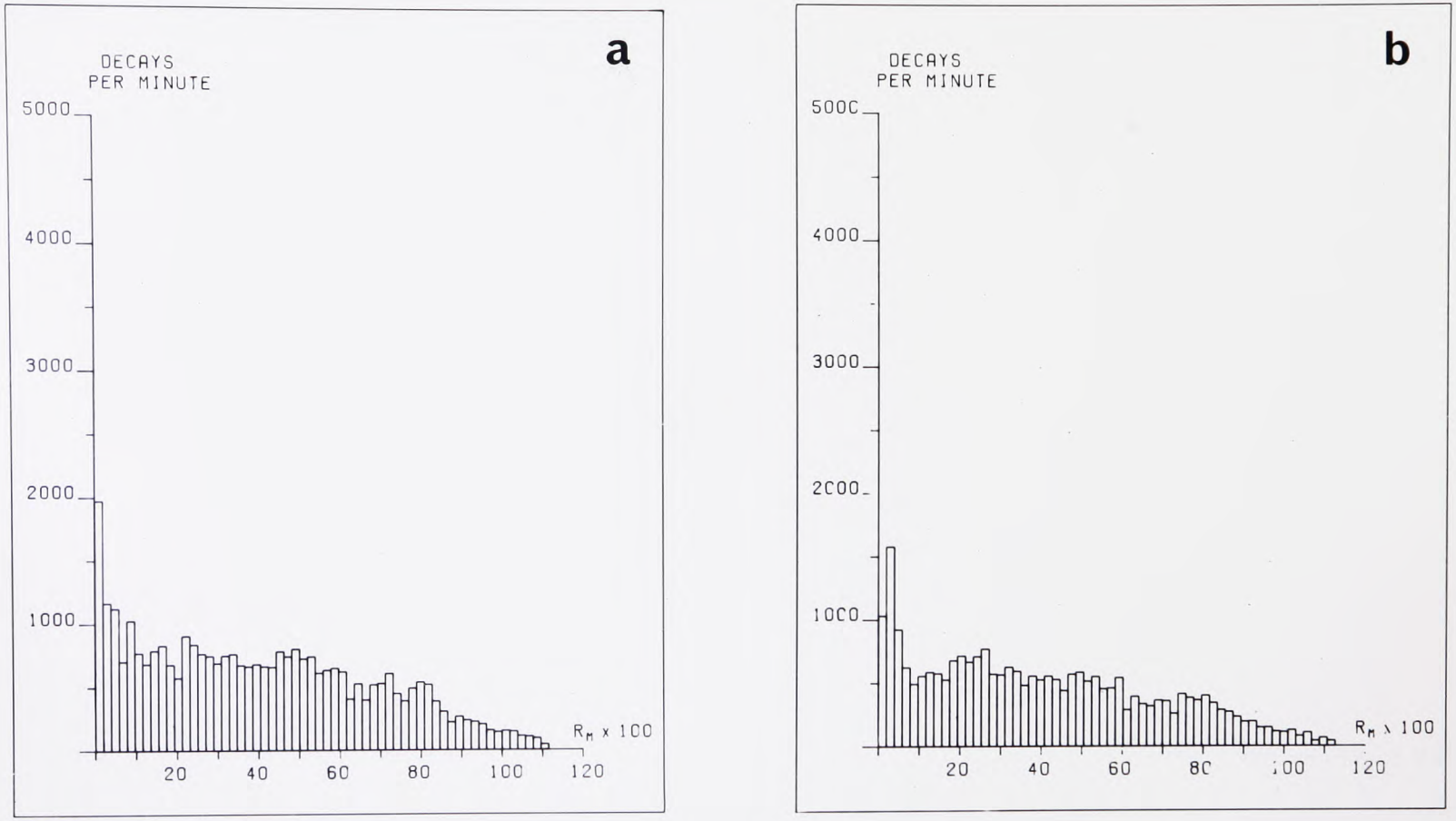


Fig. [illegible]. ^{14}C-leucine Labelling-patterns from Resting and Carbachol-Stimulated Denervated Ganglia. The [illegible] labelled for 2.5 hours, after a pre-incubation period of [illegible] hours. (a). Resting ganglia. (b). Ganglia stimulated with Carbachol (final concentration 20 microgrammes per ml) during the pre-incubation.

In the third and fourth experiments in this series, the ganglia used for labelling had been chronically denervated in vivo, by cutting the pre-ganglionic nerve fibres 12 days before surgical removal of the ganglia from the rats. By comparison with other profiles, the pattern from unstimulated, denervated ganglia (Fig. 6.13.a, recovery of radioactivity: 57.0%) appeared rather flat in the region 20 - 45, where patterns from normal ganglia (see Fig. 6.3) display a characteristic feature of a peak at 25, a smaller peak at 35, a pronounced trough at 40 and another peak at ca. 43. Although the earlier component (at ca. 22) of the first peak can still be seen in the pattern from the denervated ganglia, the prominent peak at 25 has vanished and the trough at 40 appears "filled-in".

For the final experiment in the series, denervated ganglia were stimulated by including carbachol (final concentration: 20 microgrammes per millilitre) in the pre-incubation medium throughout the 4-hour period. The profile still appears rather flat, however the peak at 25 has re-appeared. While the profiles themselves (Figs. 6.13 a and b) may seem to afford only rather tentative evidence for suggesting that this component at $R_m \times 100 = 25$ is "responding" to a "stimulus", since the change in peak-height is rather small, this is the only readily-apparent alteration in the profile between the two patterns and it falls in an area in which there is a discernable difference from the patterns given by normal ganglia.

Table 6.5. Electrophoretic Properties of Rat Adrenal Chromaffin Granule and Ganglion Proteins.

Rat Adrenal Chromaffin Granules. (a) Soluble Proteins.			Rat Adrenal Chromaffin Granules. (b) Membrane Proteins.			Rat Ganglia. Total Proteins.	
Mean R_m x 100.	SEM(n).	Staining.	Mean R_m x 100.	SEM(n).	Staining.	Mean R_m x 100.	Staining.
						3.105	F
			6.43	- (1)	VF	6.885	F
			7.643	0.573 (3)	VF		
			12.733	0.594 (3)	F	11.290	VF
			14.740	0.739 (3)	F/M	13.410	VF
16.21	- (1)	VF				15.660	F/M
			17.357	0.665 (2)	VF	17.070	F
						19.115	M
						20.746	VF
			22.325	0.695 (2)	VF		
23.59	0.000 (2)	F	23.843	0.633 (3)	F/M		
26.910	0.170 (2)	F				25.513	VF
						28.480	M
			31.263	0.440 (3)	VF		
32.03	0.180 (2)	S	33.343	0.540 (3)	VF	32.887	VF
36.740	0.180 (2)	M	37.155	0.585 (2)	VF	36.100	M
			39.967	0.620 (3)	VF	38.915	VF
40.890	- (1)	VF	42.67	- (1)	VF		
						44.608	F
46.175	0.024 (2)	VF	46.460	0.622 (3)	M	46.690	M/S
			50.807	0.521 (3)	M/S	49.935	F
53.210	0.530 (2)	VF				53.480	F
			57.760	0.371 (3)	F	58.360	VF
60.94	- (1)	VF	60.910	0.359 (3)	VF	61.515	M
			66.857	0.176 (3)	S	66.240	F
						69.170	F
73.070	0.250 (2)	VF	73.753	0.322 (3)	M	74.375	F
			77.67	- (1)	VF	78.167	VF
			83.847	0.095 (3)	F		
86.840	0.340 (2)	VF				85.653	VF
			92.760	0.278 (3)	S	93.295	M/S
			97.923	0.601 (3)	F	97.425	F
			101.997	0.352 (3)	M	101.200	M

Symbols:- VF: very faint, F: faint, M: medium, S: strong.

Electrophoresis of Rat Adrenal Chromaffin Granule Proteins - Comparison with Superior Cervical Ganglion Staining Patterns.

In order to discover whether any of the stained bands in the electrophoretic patterns of SDS-dissolved superior cervical ganglion proteins could be shown to be identical with proteins from adrenergic vesicles from a different tissue, Paggi and Toschi prepared a small quantity of rat adrenal chromaffin granules (by differential and discontinuous density-gradient centrifugation through 1.6M sucrose, as described for bovine adrenal chromaffin granules by Smith & Winkler, 1967b). After transportation to Oxford, the granules were lysed with 0.005M tris-sodium succinate buffer, pH 5.9, as described for bovine granules in an earlier chapter, to give soluble and insoluble (membrane) protein fractions, which were treated with SDS and submitted to electrophoresis on 6% polyacrylamide gels in the same way as the ganglion proteins. The mean relative mobilities of the soluble and insoluble rat adrenal chromaffin granule proteins, and their relative staining intensities, are set out in Table 6.5, together with the mean relative mobilities of rat superior cervical ganglion proteins (from Table 6.2).

There are a number of similarities between the three sets of $R_m \times 100$ values which could be taken as tentative evidence for assuming identity between various ganglion and adrenal proteins, establishing their origins as "neurovesicular", but it is clear that the ganglion pattern contains a number of major components (for example, those with mean relative mobilities of 19.115, 28.480 and 69.170) which do

not obviously correspond to either soluble or insoluble adrenal proteins. Furthermore, in most cases the prominent components of the adrenal protein patterns correspond to bands in the ganglion pattern which are noted only as "faint", and a large proportion of the staining in the ganglion pattern is probably due to proteins unrelated to those comprising secretory granules.

The most striking and obvious case of identity between ganglion and adrenal proteins, however, is with the three fastest-running components of the ganglion pattern, which clearly correspond with the three fastest-running insoluble (membrane) adrenal chromaffin granule proteins. This is, in a sense, unfortunate, since the extent of radioactive labelling is particularly low in the high-mobility ganglion proteins, being much more pronounced in the low-mobility region where the correspondence between ganglion and adrenal proteins is less easily established, but it does show that there are some proteins at least which are common to both tissues.

DISCUSSION

The Gel-slicing Methodology.

Variations in the intraganglionic concentration of some particular protein can only be measured and related to the past history and physiological state of the ganglion if some method exists which permits that protein to be detected selectively, sensitively and quantitatively. If the protein has an enzymic activity, this activity may be used as a "marker" for the protein and many studies of the rates of "axoplasmic flow" (flow within nerve axons, predominantly from perikaryon to terminal) of enzymes which participate in the biosynthesis of the neurotransmitter, noradrenaline, (see review by Dahlström, 1971) and of the activities of these enzymes in ganglia and other tissues, after drug treatment or surgical manipulation (see inter alia: Thoenen et al., 1971; Thoenen et al., 1972; Hendry et al., 1973; and review by Thoenen, 1972) make use of this approach. However, the observed increases or decreases in enzymic activity may be due to changes in the concentrations of activators or inhibitors and the results of these experiments can only be interpreted in terms of alterations to the rate of synthesis of the enzymes if such effects as variation in the activity of the enzyme have been shown to be absent.

We chose not to use an enzymic method for the studies described in this chapter, since most of the proteins making up chromaffin granules, and presumably therefore neurosecretory granules, have no

known enzymic activities. We chose instead to separate the solubilised proteins by electrophoresis in polyacrylamide gels, regarding their relative mobilities as their identifying ("marker") properties. This approach has the advantage of displaying all of the ganglion proteins in a single experimental profile when changes in their relative prominences may be clearly seen, without the interpretational problems associated with intercomparison of quantities of components detected by different methods. In principle, if the proteins are clearly resolved from one another, this method should permit changes in the amounts of individual proteins to be followed (although their specific activities, and hence their absolute concentrations, are unknown and could only be determined if sufficient material were available for their isolation in pure form) and will also demonstrate the distribution of radioactive label amongst proteins of different molecular weights.

The method chosen for analysing the labelling-profiles in the electrophoretic gels was based on that described by Loening (1967), in which the gels are mechanically sectioned into 1.0mm lengths. This is preferable to the technique described by Maizel (1966), since the fraction number may be correlated with migration distance in the gel (and hence converted to relative mobility), whereas this correlation is much less certain with the extrusion method and most authors using such a method plot their profiles as radioactivity versus fraction number.

In my experiments, the electrophoretic separation, scintillation-

counting and computational stages of the analysis were found to be highly reproducible and subject only to small, random errors, but the efficiency of chemical extraction of the radioactivity, as reflected in the overall recoveries, was found to vary from one batch of Soluene to the next. The makers (Packard Instruments Ltd.) have strenuously denied the suggestion that there may be some variation in the quality of their product, but it is hard to find an adequate explanation for the sudden drop in recovery noted in four experiments which all made use of one batch of Soluene (not used for any other experiment) without implicating this chemical, since no other change in conditions or procedure was recorded. Under favourable circumstances, however, the recovery of radioactivity from the gel was in the range 80-90% for ^{14}C and 70-75% for ^{3}H, which agrees quite well with reports by other investigators (e.g.: Gray & Steffensen, 1968, who quote 88% recovery for ^{35}S - which may be considered equivalent to ^{14}C in scintillation-counting terms - and 75-80% recovery for ^{3}H) but does not agree with the statement of Karlsson & Sjöstrand (1971a) that the recovery of radioactivity with this method was quantitative.

The major errors in the labelling-patterns stem from the process of section-cutting and may be divided into random errors and systematic errors. The random errors arise from the variation in section-thickness and this has been estimated to be $\pm$ 4.58% for 1.0mm slices (see Table 6.3). The systematic errors are revealed as spurious peaks or troughs

resulting from the addition of dry ice with concomitant re-freezing and thawing at various points during sectioning, or in variation in the detailed shapes of duplicate profiles, which can be attributed to the effect of uncertainty in the thickness of the first slice. It has already been shown, from a consideration of a theoretical model of the sectioning process, that variations in detailed peak-shape are a distortion which is inherent in the method chosen for sampling the (continuous) labelling-profile, and such distortion must be present in patterns reported by other workers, although its seriousness will depend on a combination of experimental factors. Its importance may be expected to diminish as the separation between peaks is increased (which may be achieved by using longer polyacrylamide gels, run for a greater time) or as the thickness of the sections is reduced (which much increases the labour involved in performing the experiment).

The latter expedient was adopted by Gray & Steffensen (1968) who cut 0.25mm sections from acrylamide gels after they had been dehydrated and embedded in paraffin, however this has the inevitable result of reducing the dpm per slice, which will, in turn, reduce the accuracy with which the radioactivities may be determined by scintillation-counting, even if inconveniently long counting-times are used. If the sample contains only a small amount of labelled protein, a diminution of the slice-thickness may reduce the radioactivity in the peaks almost to background level (see, for example, Karlsson & Sjöstrand, 1971 a, c & d, where, even with 1.0mm slices, the major peaks in some

of the profiles are only 50 cpm and no indication is given of the accuracy with which the radioactivities were determined).

The only way of overcoming the problems associated with sectioning gels is to use a method of detecting the labelling-profile which gives a truly continuous record of dpm versus distance. Only one such method has been described (Tykva & Votruba, 1972) and this involves moving a thin slice of the gel, *in vacuo*, beneath a small semiconductor device which detects the emitted electrons (β-particles). Unfortunately, the method, as so far developed, is of relatively low sensitivity (detection efficiency ca. 6% for ^{14}C), cannot be used for counting tritium and has limited longitudinal resolution, so it cannot be regarded as a satisfactory alternative to the kind of gel-slicing and scintillation-counting method described here, which still represents the best available compromise between experimental convenience and precise measurement of the labelling-profile.

Labelling-patterns and Factors Affecting Protein Synthesis in Rat Superior Cervical Ganglia.

The earlier parts of this chapter have included figures showing a variety of labelling-patterns obtained under a number of different experimental conditions, and it has been commented that the patterns show certain consistent features which do not appear to vary greatly from one pattern (and one set of experimental conditions) to another. If this is so, must it be concluded that the patterns are not

representative of newly-synthesised neurovesicular proteins, but instead show up other proteins which are unrelated to the secretory activity of the ganglia, or that the conditions of labelling were unsuitable for the detection of changes in synthesis of these (neurovesicular) proteins? Must it, indeed, be concluded that the isolated superior cervical ganglion is not an useful experimental system for studies of this kind?

It was recognised at the outset of these studies that some interesting questions could not be answered, because of the limitations of the experimental circumstances. For example, it was not possible to determine the sub-cellular distribution of newly-synthesised proteins in ganglia because the amount of tissue available was too small, and in our experiments the ganglion cell has had to be treated as a whole. Other authors have chosen to work with tissues which are available in sufficient quantity for this not to be a limitation. Thus, Winkler et al. (1972) found that 4 hours after injection of ^{3}H-leucine into isolated, perfused bovine adrenal medulla, labelled soluble proteins could be recovered in a particulate fraction which had similar properties to chromaffin granules when submitted to density-gradient centrifugation; whereas at shorter time intervals after labelling, the bulk of the particle-bound soluble protein was found in a lighter, microsomal fraction. The insoluble proteins, corresponding to the membrane, of the chromaffin granule-like particle were not detectably labelled at any time interval studied

and labelled insoluble proteins were always found to be confined to the light microsomal membrane fraction. Working with chopped guinea-pig cerebral cortex, Jones and Banks (1970a) were able to show that electrical stimulation caused an alteration in the distribution of total radioactivity between nuclear, microsomal, mitochondrial and supernatant fractions, and that, at all time periods studied (up to 30 minutes after administration of the radioactive amino acid), stimulation increased the contribution made by proteins of the nuclear fraction to the total radioactivity of the whole cell homogenate. (Their finding that, in all fractions, the incorporation of label into protein was diminished by electrical stimulation will be discussed later.)

The mammalian superior cervical ganglion is, in fact, a relatively complicated structure, both in its morphology and biochemical functioning (see inter alia: Koelle, 1963; Matthews & Raisman, 1969; and Jacobowitz, 1970). It is thought that the incoming pre-ganglionic cholinergic nerves form synapses not only with the ganglion cell body itself, but also with dopaminergic interneurons (Laverty & Sharman, 1965; Björklund et al., 1970; Libet, 1970; see also Fig. 1 of Greengard & McAfee, 1972) which exert an inhibitory effect on the generation of action-potentials, recorded from the outgoing (post-ganglionic) nerve axons. Additionally, the efferent axons are surrounded by glial cells and some of the newly-synthesised proteins seen in labelling studies could have their origin in this (non-neuronal)

source. Furthermore, rat superior cervical ganglion shows a high rate of oxygen and glucose uptake (Larrabee & Klingman, 1962), characteristic of a high general metabolic rate. Does this structural and biochemical complexity mean that the specific synthesis of neurovesicular protein will be obscured by the back-ground "noise" of non-specific protein synthesis, occurring in locations other than the cell body of the ganglion or in organelles other than newly-formed secretory vesicles?

To answer this question, we must turn to the measurements of the relative mobilities of the soluble and insoluble proteins of rat adrenal chromaffin granules set out in Table 6.5 and to the labelling patterns from intact, resting ganglia. If the situation in the rat ganglion is analogous to that pertaining in bovine adrenal chromaffin cells, we may expect, from the results of Winkler et al., (1972), that the soluble proteins of the secretory vesicles in the ganglia will rapidly become labelled, while the membrane proteins of the vesicles will be only feebly radioactive. If this is the case, and if this vesicular protein accounts for a major proportion of the newly-synthesised protein in the ganglia, we may expect that the labelling-pattern will show peaks of radioactivity at mobilities coinciding with those of the soluble proteins from rat adrenal chromaffin granules, rather than with those of the insoluble (membrane) proteins.

Is this predicition borne out on examination of the profiles

from intact, resting ganglia? Although it is difficult to banish the "eye of faith" entirely when making such judgements, inspection of profiles such as those of Fig. 6.3 and Fig. 6.10 has led me to conclude that the hypothesis is substantially correct. The peaks in the profiles do appear to lie at the mobility values expected for the soluble proteins, although there is not an exclusive 1:1 correspondence (which must indicate that there are proteins located outside the soluble protein phase of the secretory vesicles which are nevertheless rapidly synthesised), and the "match" between radioactive peaks and stained bands of soluble (chromaffin granule) proteins is perhaps most clearly seen in the profile obtained with a 20-minute labelling-period (Fig. 6.11).

If, then, it appears that the labelling-patterns do indeed largely represent newly-synthesised neurovesicular protein, why does the rate of synthesis of this protein appear to be affected to only a rather limited extent by manipulations such as electrical stimulation or surgical transection of the pre-ganglionic nerves? It may be that the small response observed is a consequence of the limited time-scale of the experiment. It is by now well established that continuous and prolonged stimulation of the superior cervical ganglion (and other innervated tissues) leads to an increase in the synthesis of some of the enzymes involved in the biosynthesis of noradrenaline, and this phenomenon has been named "trans-synaptic induction". This induction of enzyme

synthesis has been found to be limited to two of the enzymes in the pathway to noradrenaline (Thoenen et al., 1971) - tyrosine hydroxylase (which catalyses the rate-determining conversion of tyrosine to dopa) and dopamine β-hydroxylase (which carries out the final reaction in the sequence, producing noradrenaline from dopamine) - while other enzymes, such as dopa decarboxylase and monoamine oxidase (one of the principal degradative enzymes, localised in mitochondria, responsible for the oxidative de-amination of noradrenaline which leads to its pharmacological inactivation), do not increase (Thoenen et al., 1971; Hendry et al., 1973). Trans-synaptic induction is, however, a relatively long-term phenomenon, since it takes approximately 4 days for the level of tyrosine hydroxylase in rat superior cervical ganglia to reach 200% of the control level, on exposure of rats to cold-stress, after which period the dopamine β-hydroxylase level had risen to only 131% of the control value. (The smaller increase noted for dopamine β-hydroxylase is probably a reflection of its higher rate of turnover, which has been estimated (Thoenen et al., 1971) to have a half-time of 13 hours.) Thus, it seems unlikely that there will be a significant increase in protein synthesis brought about by the mechanism of trans-synaptic induction during the relatively short periods of labelling and stimulation used in our studies.

The small response of labelling to stimulation that was observed in our experiments may also be due to a counterbalancing

of a number of factors which can all affect the quantity of protein synthesised and its specific activity. A wide range of changes in levels of a great variety of molecules have been found to accompany nerve stimulation and their inter-relationships and possible effects on protein synthesis are still little understood. The extremely rapid changes in concentration of various ions which accompany the propagation of action-potentials will not be taken into account in discussing our studies, but some relatively rapid processes may be important.

For example, McAfee et al. (1971) found that within 1 minute of beginning supramaximal electrical stimulation (10Hz) of rabbit superior cervical ganglia the concentration of cyclic AMP in the stimulated ganglia had increased to 500% of the content of control (unstimulated) ganglia and that the increased level was maintained for at least 8 minutes. Furthermore, if acetylcholine receptors on the ganglion cell body were blocked with hexamethonium and atropine, no increase in cyclic AMP content followed stimulation of the pre-ganglionic nerves, even though acetylcholine release continued. Another interesting finding, in view of the presence of dopaminergic interneurons in the superior cervical ganglion, was the observation by Kebabian & Greengard (1971) that the application of exogenous dopamine greatly increased the ganglionic content of cyclic AMP even in the absence of electrical stimulation, 10 μM dopamine giving a level of 500% of control (untreated) ganglia

in the presence of theophylline (a phosphodiesterase inhibitor). Cyclic AMP has not been shown to have an effect on ganglionic protein synthesis and it has been suggested (Greengard & McAfee, 1972) that its role is that of a modulator of the processes leading to hyperpolarisation of the ganglion cell body (which results in the generation of an action-potential), but the significance of these studies lies in the demonstration that profoundchanges in the chemical environment of the ganglion may follow very closely on the onset of electrical stimulation, and that these changes may persist over a significant time-period.

Of much more immediate concern to our labelling experiments was the demonstration by McBride & Klingman (1972), in a paper which was published at about the time when our experiments were in progress, that the intraganglionic concentrations of free amino acids showed considerable fluctuations during incubation and that changes in the levels of amino acids in resting ganglia differed from the changes seen in ganglia which were electrically stimulated. Using a sensitive gas-liquid chromatographic method, McBride & Klingman measured the levels of 13 amino acids in resting and stimulated rat superior cervical ganglia, at incubation times of 0.5, 1.0, 2.0 and 4.0 hours following an initial 0.5-hour pre-incubation. (See Table 1 of their paper, parts of which are reproduced below). While in most cases the concentration of the free amino acid did not change very much in resting ganglia, there was a

marked drop after 1.0 hour in the concentrations of valine, leucine, isoleucine and proline and a rise in the content of serine and glycine.

Table 6.6.

Free Amino Acid Concentrations in Rat Ganglia (from Table 1 of McBride & Klingman, 1972).

Amino Acid.		nmol/mg wet wt. after incubation for 0.5 hr.	1.0 hr.	2.0 hr.	4.0 hr.
Serine	(R)	2.0 ± 0.3	2.8 ± 0.4	2.4 ± 0.5	3.9 ± 1.0
	(S)	1.2 ± 0.3	3.2 ± 0.4	5.8 ± 1.4	3.4 ± 0.8
Alanine	(R)	1.9 ± 0.1	1.9 ± 0.2	2.2 ± 0.4	3.4 ± 0.5
	(S)	2.9 ± 0.3	2.4 ± 0.1	5.4 ± 0.8	3.0 ± 0.5
Leucine	(R)	0.60 ± 0.09	0.14 ± 0.01	0.62 ± 0.13	0.50 ± 0.09
	(S)	0.44 ± 0.06	0.35 ± 0.03	0.70 ± 0.10	0.60 ± 0.13
Valine	(R)	0.50 ± 0.06	0.17 ± 0.04	0.31 ± 0.09	0.60 ± 0.10
	(S)	0.41 ± 0.07	0.31 ± 0.04	0.90 ± 0.21	0.74 ± 0.10
Threonine	(R)	0.81 ± 0.07	0.78 ± 0.08	0.80 ± 0.22	0.73 ± 0.20
	(S)	0.82 ± 0.11	0.76 ± 0.05	1.80 ± 0.40	0.81 ± 0.24

Data are given as means ± SEM of 4-6 determinations for resting (R) and stimulated (S) ganglia.

The most striking change, however, was seen after 2.0 hours of incubation, when there was a large percentage rise in the concentrations of alanine, serine, glycine, valine, isoleucine, leucine, threonine, proline and phenylalanine, the concentrations being 326% (isoleucine), 290% (valine), 237% (threonine), 207% (proline) and 200% (leucine) of the corresponding values at 1.0 hours. After 4.0 hours, the free amino acid levels in both resting and stimulated ganglia had mostly returned to values near to those found after 0.5 hours, although there are exceptions to this generalisation (e.g.: serine and glycine and, to a lesser extent, valine) and when the figures are examined in detail it can be seen that each amino acid exhibited a slightly different pattern of fluctuation in concentration versus time.

McBride & Klingman showed that the amino acid composition of the total TCA-precipitable protein was not significantly affected by electrical stimulation (although changes could have occurred in the amino acid compositions of individual proteins without detectably altering the composition of the total proteins), whereas the protein content of stimulated ganglia was somewhat lower (ca. 10%) than that of control ganglia. They also studied the effects of stimulation on the sodium-dependent efflux of amino acids from the ganglia and reported that while the incorporation of label from ^{14}C-glutamate into total TCA-insoluble protein was

higher, over the course of 1.0 hour, in resting ganglia than in stimulated ganglia, this difference vanished if the incubation medium was supplemented by the addition of (unlabelled) amino acids in concentrations comparable to those found in plasma. Although their measurements are not beyond criticism (for example, they made no attempt to discover whether a significant proportion of the total amino acid content of the ganglion is extracellular, treating all of the amino acid detected as being localised intracellularly), McBride & Klingman provided valuable data for all investigators interested in using isotopic tracer methods to follow protein synthesis in this tissue, since their results indicate that the choice of labelled amino acid may be critical in determining the response of apparent incorporation to experimental stimuli. (For example, it might be preferable to use radioactive phenylalanine in place of the more common leucine or valine, because of the smaller variation in its concentration within ganglia and despite the fact that phenylalanine residues may be less abundant in proteins than leucine or valine residues).

It seems very likely that our experiments will have been affected by fluctuations in the intraganglionic concentration of free leucine, since the labelling extended over a period of 2.5 hours, normally following a pre-incubation of 30 minutes. During the labelling-period, then, the leucine concentration might be expected to drop considerably (see: Table 6.6) and then recover,

but we have no data to show how the specific activity of the leucine pool in the ganglion changes during this time. McBride & Klingman do not attempt to explain how the observed changes in amino acid levels may be caused, and it is not clear whether, for example, the drop in the content of several amino acids, including leucine and valine, which occurs after the first hour of incubation is a result of changing the bathing medium at the end of the pre-incubation period. If it is, does such a drop occur whenever the medium is changed? And does its magnitude depend on the length of time that has elapsed since the ganglia were removed from the rat? Unfortunately, McBride & Klingman did not investigate these possibilities.

Such questions may critically affect the interpretation of the experiments we performed with stimulated and/or denervated ganglia (Figs. 6.12 a and b and 6.13 a and b), since a 4-hour pre- incubation was adopted for these experiments, instead of the usual 30-minutes. If there was no drop in leucine level when the medium was changed, after 4 hours, and instead the concentration remained constant for the following 2.5 hours, then the average intraganglionic leucine concentration throughout labelling would be higher in these experiments than in others in which ganglia were pre-incubated for 30 minutes. If this, in turn, means that the specific activity of the leucine was lower after the longer pre-incubation, then the total incorporation of label into protein will

also be lower, even though the labelling was carried out under experimental conditions which were as nearly as possible identical. Some preliminary experiments tend to support this idea (G. Toschi, personal communication), although the effect is probably not very great (ca. 20%).

It was remarked (see under "results") that, if the profiles from the two experiments showing the effect of electrical stimulation (Figs. 6.12 a & b) were considered in isolation, then electrical stimulation appeared to have brought about some increase in the synthesis of proteins migrating with R_m x 100 in the region 10-35. How does this compare with the conclusion of Banks (1970) that, in the guinea-pig superior cervical ganglion, protein synthesis is inhibited by electrical stimulation over the time period of several hours, although there was a transient increase compared with the control over the first few minutes? Jones and Banks (1970a) had demonstrated a similar effect in chopped guinea-pig cerebral cortex and had gone on to perform experiments on the fluxes of labelled amino acid (valine) in this tissue which led them to suggest that the initial enhancement of synthesis was probably due to a short-lived increase in the rate of valine uptake (see Jones & Banks, 1970b). The extent of this enhancement in synthesis was found to be dependent on the frequency of stimulation, if incorporation was measured over the first 3.5 minutes, and although synthesis was not increased over the control (unstimulated)

value if carried out at 5 Hz, it rose by 24.8% at 10Hz (the frequency used in our studies), by 50% at 20 Hz and by 85.6% at 100Hz. Interestingly, the inhibition of synthesis which was noted after stimulation for 15 minutes at 100Hz did not occur if the frequency was lowered to 20Hz. In studies on superior cervical ganglion, however, (Banks, 1970) inhibition of synthesis was noted even at a frequency of 4Hz, reaching a level value 30% below the rate of synthesis in unstimulated control ganglia within 1 hour of the commencement of stimulation.

Although there are many possible points of control in the metabolic processes occurring within nervous tissue and many possible mechanisms which might bring about this observed inhibition of synthesis, Jones and Banks (1970a) considered that depletion of high-energy phosphates might be important in this respect and they were able to show that the levels of both adenosine triphosphate (ATP) and creatine phosphate fell after electrical stimulation, and Banks (1970) showed a similar drop in the ATP content of ganglia. It is interesting to recall in this connection that the effect of dopamine in increasing tissue content of cyclic AMP, referred to earlier in this discussion, was due to the activation of the enzyme which produces cyclic AMP (adenyl cyclase) and that this enzyme uses ATP as its substrate. While this may be fortuitous, or not of physiological significance, it may be an indication of the integration of the metabolic, excitatory and synthetic functions

of the ganglion cell body.

In conclusion, then, it seems that our understanding of the processes governing the levels of important metabolites and ions in ganglia, and of the topochemical aspects of ganglion functioning is as yet insufficiently comprehensive to allow a detailed interpretation of protein-synthesis studies of this kind, in which the observed changes in labelling are rather subtle. In particular, it is necessary to know much more about not just the intraganglionic concentration of the amino acid being used to label protein, but also its *in situ* specific activity and its distribution throughout the morphologically distinct areas of the cell. There remains also the problem of "back-ground noise". If it is desired to study those proteins which are transported rapidly down the nerve axon, one possible way of reducing this interference from non-specific synthesis would be to administer radioactive label to the ganglion cell body and then observe the accumulation of rapidly-flowing proteins above a ligation in the outgoing axon, possibly using the kind of double-compartment chamber described by Banks et al.. (1971) in their experiments with the inferior mesenteric ganglion/ hypogastric nerve preparation. It is reasonable to hope that significant advances in our knowledge of protein synthesis in ganglia could follow from the application of present analytical techniques to the study of samples which have been "cleaned up" in this physiological fashion.

APPENDIX

The three computer programs (OVERPLOT, GAUSSCALC and CHOP) used during the gel-slicing experiments described in chapter 6 of this thesis were all written in the ICL 1900 Algol language, which is an enhanced implementation of the officially-defined Algol 60 reference language and which was designed by ICL for use on their 1900 - series computers. All of the programs make use of graphical procedures from the (local) OXGHOST graph-plotting procedures library and they were designed and operated under the GEORGE 3 operating system of the University's 1906A computer (using Marks 6.6 and 7.5 of GEORGE 3). They are included here as "fold-out" line-printer output, rather than as a re-typed text, since it was felt that the structures of the programs would be more easily understood in this "continuous" format, and because this is the appropriate hardware representation for card-punching, which includes some symbols not available on a typewriter.

When the programs were written, no special effort was made to ensure the most efficient use of filestore or central processor time, and they were regarded merely as "tools" for use in the analytical technique. From the programming point of view, they are probably far from being the most "elegant" representation of the algorithms. All of the binary programs produced from them by the 1906A Algol compiler are small, however, (less than 10 K words) and execution times with normal data did not exceed 20 seconds of

total job time (central processor time being only a relatively small part of this time). None of the programs make use of highly-specialised programming methods, nor was it necessary to include segments in PLAN (the ICL assembly-language), and only GAUSSCALC requires more than the standard single input and output channels (provided as default options by the compiler). Since the programs all produce graphical output in the same format, large sections of these three programs are very similar - partly a consequence of the fact that all external (library) procedures must be declared explicitly before use in Algol programs. The small differences between the programs are however, important, as they determine the exact appearance of the final graphs.

GAUSSCALC produces two different kinds of output: a file of numbers (which are to be used as input data by the program CHOP) and a more informative listing, which is usually sent to a file which may subsequently be printed, using a line-printer. It is necessary to include a program description segment before the program itself, to advise the compiler of this distinction, and this is seen as the first six lines of GAUSSCALC, before the first 'begin'. (This segment also makes provision for a post-mortem dump, if the program should fail during execution. Note: the program description segment at the beginning of CHOP is not strictly necessary and may be omitted, as in OVERPLOT).

The separation between the two output streams must also be

declared explicitly at the operating-system level, by using GEORGE command-language. Thus the sequence of commands:

```
OXCOMP DATA *CR datafile,*GP plotfile,*LP outfile1,-
#(AS *LP1,outfile2)
*ALGOL *CR GAUSSCALC
*LINK LIB OXGHOST
*DATA BIN binfile
*END
LF outfile1,*LP
ENDJOB
```

will cause the Algol program GAUSSCALC to be compiled, with the inclusion of segments from the OXGHOST library, and a copy of the resulting binary program will be stored in the file "binfile" (any string of up to 12 alphanumeric characters may be used as the identifying name for a file, and such a name (chosen by the user) is indicated in this example by the use of lower-case letters). The binary program will then be executed, using data stored in "datafile", producing output in line-printer format in "outfile2" (the output to be used as data by CHOP) and a file of graph-plotting instructions in "plotfile". Finally, the contents of "outfile1" are printed out, using a line-printer, and the job ends. At this stage, the computer will <u>not</u> produce any graph-plotting, since this is a two-stage process and "plotfile" contains only the instructions necessary to produce a plot. To produce the graphical output on the incremental plotter, the user must then give the command:

OXPLOT OXGHOST plotfile,*GP

when the plotting will be done. Although the commands in these examples are only valid on an ICL computer using the GEORGE 3 operating-system, it is hoped that they make clearer the inter-relation between the various items of transput associated with these programs.

```
#OUTPUT BY LISTFILE IN '|BARTLETT,MUPFORTHESIS' ON 28AUG73 AT 23,01,18

DOCUMENT     CHOP


'LIST' (LP)
'PMD' (ED,CHUPFLUP)
'PROGRAM' (AXXX)
'INPUT' 0=CRU
'OUTPUT' 0=LPU
'BEGIN'
  'COMMENT' THIS INCREMENTAL CHUPPING PROGRAM TAKES OUTPUT FROM STREAM
  ONE OF THE GAUSSIAN SIMULATION PROGRAM,AFTER THE APPROPRIATE VALUES
  FUR THE DYE MIGRATION DISTANCE AND THE NUMBER OF SLICES HAVE BEEN
  ADDED TO IT BY EDITING THE OUTPUT FILE, IT PRODUCES TEN HISTOGRAMS,
  AT INTERVALS OF 0,1MM,STARTING WITH NO INCREMENT;
  'REAL' SUM,A,B,DYE,INC;
  'INTEGER' UPPER,LUWER,POTS,PEAKS,J,K,P;
  'REAL' 'ARRAY' BIT,ORD[1:1200];
  PEAKS:=READ;
  'FOR' J:=1 'STEP' 1 'UNTIL' 1200 'DO'
     'BEGIN'
     BIT[J]:=READ;
     ORD[J]:=READ;
     'END';
  POTS:=READ;
  DYE:=READ;
     'BEGIN'
     'INTEGER' N;
     'REAL''ARRAY' HT[1:POTS];
     'PROCEDURE' FRAME;'EXTERNAL';
     'PROCEDURE' BORDER; 'EXTERNAL';
     'PROCEDURE' POINT(X,Y); 'VALUE' X,Y; 'REAL' X,Y; 'EXTERNAL';
     'PROCEDURE' JOIN(X,Y); 'VALUE' X,Y; 'REAL' X,Y; 'EXTERNAL';
     'PROCEDURE' GREND; 'EXTERNAL';
     'PROCEDURE' PLOTAS(X,Y,S);
     'VALUE' X,Y;
     'REAL' X,Y;
     'STRING' S;
     'EXTERNAL';
     'PROCEDURE' PLOTNF(X,Y,Z,NDP);
     'VALUE' X,Y,Z,NDP;
     'REAL' X,Y,Z;
     'INTEGER' NDP;
     'EXTERNAL';
     'PROCEDURE' PLOTNI(X,Y,I);
     'VALUE' X,Y,I;
     'REAL' X,Y;
     'INTEGER' I;
     'EXTERNAL';
     'PROCEDURE' CRSIZE(HEIGHT);
     'VALUE' HEIGHT;
     'REAL' HEIGHT;
     'EXTERNAL';
     'PROCEDURE' LIMITS(XMIN,XMAX,YMIN,YMAX);
     'VALUE' XMIN,XMAX,YMIN,YMAX;
     'REAL' XMIN,XMAX,YMIN,YMAX; 'EXTERNAL';
     'PROCEDURE' HISTGM(XO,YO,DX,YV,M,N);
     'VALUE' XO,YO,DX,M,N;
     'ARRAY' YV;
     'REAL' XO,YO,DX;
     'INTEGER' M,N;
     'EXTERNAL';
     'PROCEDURE' REGION(XMIN,XMAX,YMIN,YMAX);
     'VALUE' XMIN,XMAX,YMIN,YMAX;
     'REAL' XMIN,XMAX,YMIN,YMAX;
     'EXTERNAL';
     'BOOLEAN' 'PROCEDURE' EVEN(X);
     'VALUE' X;
     'REAL' X;
        'BEGIN'
        'INTEGER' Q;
        Q:=X;
        'IF' ((Q/2)-(Q'/'2))>0,1
        'THEN' EVEN:='FALSE'
        'ELSE' EVEN:='TRUE'
        'END';
     'PROCEDURE' AXPLOT(X,Y,Z,P);
     'VALUE' P,X,Y,Z;
     'REAL' X,Y;
     'INTEGER' P,Z;
        'BEGIN'
        'REAL' A,B;
        'INTEGER' Q,V;
        A:=X;
        B:=Y;
        POINT(A,B);
        'IF' P=1 'THEN' JOIN(A+(Z-1)/2,B)
        'ELSE' JOIN(A,B+0,7874*(Z-1));
        'IF' P=1 'THEN'
           'BEGIN'
           CRSIZE(0,15);
           PLOTAS(1,5,9,25,'('UNITS')');
           'END'
```

```
'ELSE'
   'BEGIN'
   CRSIZE(0,15);
   PLOTAS(7,9,0,95,'('R')');
   CRSIZE(0,10);
   PLOTAS(8,05,0,90,'('M')');
   PLOTAS(8,25,0,95,'('X')');
   CRSIZE(0,15);
   PLOTNI(8,45,0,95,100);
   'END';
'FOR' Q:=1 'STEP' 1 'UNTIL' Z 'DO'
   'BEGIN'
   'COMMENT' P=1 FOR X=AXIS PLOTTING;
   POINT(A,B);
   'IF' P=1 'THEN' 'GOTO' GL1
   'ELSE'
      'BEGIN'
      'COMMENT' THIS PLOTS THE Y=AXIS;
      'IF' EVEN(Q)
      'THEN' JOIN(A=0,1,B)
      'ELSE' 'IF' Q=1 'THEN' JOIN(A=0,2,B)
      'ELSE'
         'BEGIN'
         CRSIZE(0,15);
         JOIN(A=0,2,B);
         V:=0.5*(Q-1);
         PLOTNI(A=0,4,B=0,075,V);
         'END';
      B:= B+0,7874
      'END';
   'GOTO' GL2;
   GL1:'BEGIN'
      'COMMENT' THIS PLOTS THE X=AXIS;
      'IF' EVEN(Q)
      'THEN' JOIN(A,B=0,1)
      'ELSE' 'IF' Q=1 'THEN' JOIN(A,B=0,1)
      'ELSE''BEGIN'
         CRSIZE(0,15);
         JOIN(A,B=0,1);
         V:=10*(Q=1);
         PLOTNI(A=0,15,B=0,55,V);
         'END';
      A:=A+0,5
      'END';
   GL2:'END'
'END';
'FOR' INC:=0,0 'STEP' 0,1 'UNTIL' 0,91 'DO'
   'BEGIN'
   'FOR' J:=1 'STEP' 1 'UNTIL' POTS 'DO'
      'BEGIN'
```

```
            LOWER:=B;
            SUM:=N:=0;
            'FOR' K:=LOWER 'STEP' 1 'UNTIL' UPPER 'DO'
               'BEGIN'
               SUM:=SUM+('IF' K'LE'0 'THEN' 0 'ELSE' ORD[K]);
               N:=N+1;
               'END';
            HT[J]:=SUM/N;
            'END';
         LIMITS(1.0,9.0,0.0,10.0);
         BORDER;
         LIMITS(0.0,14.0,0.0,10.0);
         AXPLOT(2,0.0,8,11,0);
         AXPLOT(2,0.0,8,13,1);
         CRSIZE(0,15);
         PLOTAS(5,0.7,0,'('INCREMENT%EQUALS')');
         PLOTNF(7,6.7,0,INC,1);
         LIMITS(2,0,8,0,0.8,9.4614);
         REGION(0,0,120,0,0.0,5.5);
         HISTGM(0,0,0,0,100/DYE,HT,1,POTS);
         LIMITS(0,0,10,0,0.0,10.0);
         FRAME;
         PAPERTHROW;
         WRITETEXT('('CALCULATED%HISTOGRAM%AT%INCREMENT=')');
         PRINT(INC,1,1);
         NEWLINE(1);
         WRITETEXT('('NUMBER%OF%SLICES:')');
         PRINT(POTS,2,0);
         NEWLINE(1);
         WRITETEXT('('DYE%RAN%TO:')');
         PRINT(DYE,2,1);
         WRITETEXT('('MM')');
         NEWLINE(2);SPACE(4);
         WRITETEXT('('SLICE%%%%%ORDINATE')');
         NEWLINE(2);
         'FOR' P:=1 'STEP' 1 'UNTIL' POTS 'DO'
            'BEGIN'
            SPACE(5);
            PRINT(P,2,0);
            SPACE(5);
            PRINT(HT[P],1,5);
            NEWLINE(1);
            'END';
         'END';
      GREND;
   'END';
'END';

↑SSSS
```

```
DOCUMENT    GAUSSCALC


'LIST' (LP)
'PMD' (ED,GAUSSFLOP)
'PROGRAM' (AXXX)
'INPUT' 0=CR0
'OUTPUT' 0=LP0
'OUTPUT' 1=LP1
'BEGIN'
'INTEGER' P;
P:=READ;
  'BEGIN'
  'REAL' X,M,SIGMA,A,B;
  'INTEGER' G,J,K,L,Q,R,S;
  'ARRAY' YMAX,MEAN[1:P],Y[1:1200];
  SIGMA:=READ;
  M:=READ;
  'COMMENT' THE STANDARD DEVIATION OF THE PTH PEAK IS TAKEN TO BE
  LINEARLY RELATED TO THE DISTANCE OF THE PEAK MAXIMUM FROM THE ORIGIN;
  'FOR' J:=1 'STEP' 1 'UNTIL' P 'DO'
     'BEGIN'
     MEAN[J]:=READ*10;
     YMAX[J]:=READ;
     'COMMENT' IN THE DATA THE MEAN IS GIVEN IN RM*100 UNITS;
     'END';
  'FOR' X:=1 'STEP' 1 'UNTIL' 1200 'DO'
     'BEGIN'
     'COMMENT' THIS SECTION CALCULATES THE ORDINATE FOR EACH VALUE OF X
     FROM RM*100=0 TO RM*100=120, IN STEPS OF 0.1 RM*100 UNITS
     BY SUMMATION OF THE CONTRIBUTIONS FROM EACH OF THE GAUSSIAN PEAKS;
     Y[X]:=0;
     'FOR' K:=1 'STEP' 1 'UNTIL' P 'DO'
     Y[X]:=Y[X]+YMAX[K]*EXP(-((X-MEAN[K])↑2)/(2*((SIGMA*M*MEAN[K])↑2)))
     'END';
     'BEGIN'
     'INTEGER' W;
     'PROCEDURE' BORDER; 'EXTERNAL';
     'PROCEDURE' POINT(X,Y); 'VALUE' X,Y; 'REAL' X,Y; 'EXTERNAL';
     'PROCEDURE' JOIN(X,Y); 'VALUE' X,Y; 'REAL' X,Y; 'EXTERNAL';
     'PROCEDURE' GREND; 'EXTERNAL';
     'PROCEDURE' PLOTAS(X,Y,S);
     'VALUE' X,Y;
     'REAL' X,Y;
     'STRING' S;
     'EXTERNAL';
     'PROCEDURE' PLOTNI(X,Y,I);
     'VALUE' X,Y,I;
     'REAL' X,Y;
     'INTEGER' I;
     'EXTERNAL';
     'PROCEDURE' CRSIZE(HEIGHT);
     'VALUE' HEIGHT;
     'REAL' HEIGHT;
     'EXTERNAL';
     'PROCEDURE' LIMITS(XMIN,XMAX,YMIN,YMAX);
     'VALUE' XMIN,XMAX,YMIN,YMAX;
     'REAL' XMIN,XMAX,YMIN,YMAX; 'EXTERNAL';
     'PROCEDURE' HISTGM(XO,YO,DX,YV,M,N);
     'VALUE' XO,YO,DX,M,N;
     'ARRAY' YV;
     'REAL' XO,YO,DX;
     'INTEGER' M,N;
     'EXTERNAL';
     'PROCEDURE' REGION(XMIN,XMAX,YMIN,YMAX);
     'VALUE' XMIN,XMAX,YMIN,YMAX;
     'REAL' XMIN,XMAX,YMIN,YMAX;
     'EXTERNAL';
     'BOOLEAN' 'PROCEDURE' EVEN(X);
     'VALUE' X;
     'REAL' X;
        'BEGIN'
        'INTEGER' Q;
        Q:=X;
        'IF' ((Q/2)-(Q'/'2))>0.1
        'THEN' EVEN:='FALSE'
        'ELSE' EVEN:='TRUE'
        'END';
     'PROCEDURE' AXPLOT(X,Y,Z,P);
```

```
'VALUE' P,X,Y,Z;
'REAL' X,Y;
'INTEGER' P,Z;
   'BEGIN'
   'REAL' A,B;
   'INTEGER' Q,V;
   A:=X;
   B:=Y;
   POINT(A,B);
   'IF' P=1 'THEN' JOIN(A+(Z=1)/2,B)
   'ELSE' JOIN(A,B+0,7874*(Z=1));
   'IF' P=1 'THEN'
      'BEGIN'
      CRSIZE(0,15);
      PLOTAS(1,5,9,25,'('UNITS')');
      'END'
   'ELSE'
      'BEGIN'
      CRSIZE(0,15);
      PLOTAS(7,9,0,95,'('R')');
      CRSIZE(0,10);
      PLOTAS(8,05,0,90,'('M')');
      PLOTAS(8,25,0,95,'('X')');
      CRSIZE(0,15);
      PLOTNI(8,45,0,95,100);
      'END';
   'FOR' Q:=1 'STEP' 1 'UNTIL' Z 'DO'
      'BEGIN'
      'COMMENT' P=1 FOR X=AXIS PLOTTING;
      POINT(A,B);
      'IF' P=1 'THEN' 'GOTO' GL1
      'ELSE'
         'BEGIN'
         'COMMENT' THIS PLOTS THE Y-AXIS;
         'IF' EVEN(Q)
         'THEN' JOIN(A-0,1,B)
         'ELSE' 'IF' Q=1 'THEN' JOIN(A-0,2,B)
         'ELSE'
            'BEGIN'
            CRSIZE(0,15);
            JOIN(A-0,2,B);
            V:=0,5*(Q=1);
            PLOTNI(A-0,4,B-0,075,V);
            'END';
         B:= B+0,7874
      'END';
'GOTO' GL2;
GL1:'BEGIN'
   'COMMENT' THIS PLOTS THE X=AXIS;
   'IF' EVEN(Q)
   'THEN' JOIN(A,B-0,1)
   'ELSE' 'IF' Q=1 'THEN' JOIN(A,B-0,1)
   'ELSE''BEGIN'
      CRSIZE(0,15);
      JOIN(A,B-0,1);
      V:=10*(Q-1);
      PLOTNI(A-0,15,B-0,35,V);
      'END';
   A:=A+0,5
   'END';
```

```
LIMITS(1,0,9,0,0,0,10,0);
BORDER;
LIMITS(0,0,14,0,0,0,10,0);
AXPLOT(2,0,0,8,11,0);
AXPLOT(2,0,0,8,13,1);
LIMITS(2,0,8,0,0,8,9.4614);
REGION(0,0,1200,0,0,0,5,5);
HISTGR(0,0,0.0,1.0,Y,2,1200);
GREND;
PAPERTHROW;
WRITETEXT('('CALCULATED%GAUSSIAN%FOR%SIMULATED%GEL-CHOPPING')');
NEWLINE(1);
WRITETEXT('('NUMBER%OF%CURVES%USED:')');
PRINT(P,2,0);
NEWLINE(1);
WRITETEXT('('VALUE%OF%SIGMA%IS:')');
PRINT(SIGMA,1,5);
NEWLINE(1);
WRITETEXT('('VALUE%OF%M%IS:')');
PRINT(M,2,6);
NEWLINE(2);
'FOR' S:=1 'STEP' 1 'UNTIL' 6 'DO'
   'BEGIN'
   WRITETEXT('('DATA%POINTS%FOR%SIMULATION%CURVE,')');
   NEWLINE(1);
   WRITETEXT('('MOBILITY%VALUES%RANGE%FROM%RM*100=')');
   PRINT((S*20,0)-19,0,3,1);
   WRITETEXT('('TO')');
   PRINT(S*20.0,3,1);
   NEWLINE(2);
   'FOR' R:=1 'STEP' 1 'UNTIL' 5 'DO'
   WRITETEXT('('%RM*100%%ORDINATE%%%')');
   NEWLINE(1);
   'FOR' R:=1 'STEP' 1 'UNTIL' 40 'DO'
      'BEGIN'
      'FOR' Q:=1 'STEP' 1 'UNTIL' 5 'DO'
         'BEGIN'
         W:=5*(R-1)+(Q-1)+200*(S-1)+1;
         PRINT(W/10,3,1);
         SPACE(1);
         PRINT(Y[W],1,5);
         SPACE(1)
         'END';
      NEWLINE(1);
      'END';
   PAPERTHROW;
   'END';
SELECT OUTPUT(1);
PRINT(P,2,0);
NEWLINE(1);
'FOR' Q:=1 'STEP' 1 'UNTIL' 500 'DO'
   'BEGIN'
   'FOR' R:=1 'STEP' 1 'UNTIL' 4 'DO'
      'BEGIN'
      PRINT((4*(Q-1)+(R-1)*1)/10,3,1);
      SPACE(1);
      PRINT(Y[4*(Q-1)+(R-1)+1],1,5);
      SPACE(1);
      'END';
   NEWLINE(1);
   'END';
'END';
'END';
'END';
```

TNNN

```
#OUTPUT BY LISTFILE IN '|BARTLETT,MUPPORTHESIS' ON CORUUT3 AT 23:03:87

DOCUMENT     UVERPLOT


'BEGIN''REAL' SLOPE,INTERCEPT,BLANK1,BLANK2,MEANBLANK,S1,S2,S3,S4,
STD1,STD2,MEANSTD,TOTSAM,RECOVERY,DECAYS,DYE;
'INTEGER' SAMPLES,STDVOL,SAMVOL,Y,N;
PAPERTHROW;
COPYTEXT('('BONK')');
NEWLINE(1);
SLOPE:=READ;INTERCEPT:=READ;
Y:=INTERCEPT;
'IF' Y=81 'THEN''GOTO' L1 'ELSE'
'IF' Y=17 'THEN''GOTO' L2 'ELSE'
'IF' Y=27 'THEN''GOTO' L3
'ELSE' 'GOTO' L4;
L1: WRITETEXT('('ISOTOPE;%14=CARBON%%%%%GATE;%14=C')');
'GOTO' L5;
L2: WRITETEXT('('ISOTOPE:%TRITIUM%%%%%GATE;%3=H')');
'GOTO' L5;
L3: WRITETEXT('('ISOTOPE:%14=CARBON%%%%%GATE;%14=C%WITH%3=H')');
'GOTO' L5;
L4: COPYTEXT('('OPTIONAL')');
L5: N:=READ;
NEWLINE(2);
DYE:=READ;
'COMMENT' DYE MUST BE IN MILLIMETRES;
BLANK1:=READ;S1:=READ;
BLANK2:=READ;S2:=READ;
STD1:=READ;S3:=READ;
STD2:=READ;S4:=READ;
BLANK1:=BLANK1/(0.01*(INTERCEPT+SLOPE*S1));
BLANK2:=BLANK2/(0.01*(INTERCEPT+SLOPE*S2));
STD1:=STD1/(0.01*(INTERCEPT+SLOPE*S3));
STD2:=STD2/(0.01*(INTERCEPT+SLOPE*S4));
MEANBLANK:=(BLANK1+BLANK2)/2;
MEANSTD:=(STD1+STD2)/2;
STDVOL:=READ;
SAMVOL:=READ;
TOTSAM:=MEANSTD*SAMVOL/STDVOL;
DECAYS:=0;
'BEGIN''COMMENT'CALCULATION OF DPM FOR EACH SAMPLE;
'INTEGER' M;
'ARRAY' S,CPM,DPM,XPT[1:N];
'FOR' M:=1'STEP'1'UNTIL'N'DO'
'BEGIN'
CPM[M]:=READ;
S[M]:=READ;
DPM[M]:=(CPM[M]/(0.01*(INTERCEPT+SLOPE*S[M])))-MEANBLANK;
XPT[M]:= (M*100/DYE)-(50/DYE);
DECAYS:=DECAYS+DPM[M]
'END';
WRITETEXT('('BLANK%1:')');PRINT(BLANK1,2,2);WRITETEXT('('DPM')');
SPACE(6);
WRITETEXT('('STANDARD%1:')');PRINT(STD1,5,1);
WRITETEXT('('DPM')');NEWLINE(1);
WRITETEXT('('BLANK%2:')');PRINT(BLANK2,2,2);WRITETEXT('('DPM')');
SPACE(6);
WRITETEXT('('STANDARD%2:')');PRINT(STD2,5,1);
WRITETEXT('('DPM')');NEWLINE(1);
WRITETEXT('('MEAN%BLANK:')');
PRINT(MEANBLANK,5,2);
WRITETEXT('('DPM')');SPACE(2);
WRITETEXT('('MEAN%STANDARD:')');PRINT(MEANSTD,5,1);
WRITETEXT('('DPM')');NEWLINE(2);
WRITETEXT('('DPM%APPLIED%TO%GEL:')');
PRINT(TOTSAM,7,1);NEWLINE(1);
WRITETEXT('('DPM%RECOVERED%FROM%GEL:')');
PRINT(DECAYS,7,1);NEWLINE(1);
RECOVERY:=DECAYS*100/TOTSAM;
WRITETEXT('('RECOVERY%OF%RADIOACTIVITY:')');
PRINT(RECOVERY,5,2);
WRITETEXT('('PERCENT')');NEWLINE(2);
WRITETEXT('('DYE%MIGRATED%')');
PRINT(DYE,2,1);
WRITETEXT('('MM')');NEWLINE(1);
NEWLINE(2); SPACE(8);
WRITETEXT('('SLICE'('6S')'CPM'('6S')'S'('8S')'DPM
'('6S')'MID-POINT')');
NEWLINE(1);
'FOR' M:=1 'STEP' 1 'UNTIL' N 'DO'
'BEGIN'SPACE(8);
PRINT(M,2,0);
SPACE(2);
PRINT(CPM[M],5,1);
PRINT(S[M],1,3);
PRINT(DPM[M],5,1);
SPACE(2);
PRINT(XPT[M],3,2);
'IF' S[M]<0,55 'OR' S[M]>0,95
'THEN' WRITETEXT('('WARNING: OUTSIDE REGRESSION RANGE')');
NEWLINE(1);
'END';
```

```
'BEGIN'
'PROCEDURE' LIMITS(XMIN,XMAX,YMIN,YMAX);
'VALUE' XMIN,XMAX,YMIN,YMAX;
'REAL' XMIN,XMAX,YMIN,YMAX;
'EXTERNAL';
'PROCEDURE' BORDER;'EXTERNAL';
'PROCEDURE' POINT(X,Y);
'VALUE' X,Y;
'REAL' X,Y;
'EXTERNAL';
'PROCEDURE' JOIN(X,Y);
'VALUE' X,Y;
'REAL' X,Y;
'EXTERNAL';
'PROCEDURE' REGION(XMIN,XMAX,YMIN,YMAX);
'VALUE' XMIN,XMAX,YMIN,YMAX;
'REAL' XMIN,XMAX,YMIN,YMAX;
'EXTERNAL';
'PROCEDURE' CURVEO(XV,YV,M,N);
'VALUE' M,N;
'INTEGER' M,N;
'ARRAY' XV,YV;
'EXTERNAL';
'PROCEDURE' HISTGM(XO,YO,DX,YV,M,N);
'VALUE' XO,YO,DX,M,N;
'ARRAY' YV;
'REAL' XO,YO,DX;
'INTEGER' M,N;
'EXTERNAL';
'PROCEDURE' FRAME;'EXTERNAL';
'PROCEDURE' GREND;'EXTERNAL';
'PROCEDURE' PLOTAS(X,Y,S);
'VALUE' X,Y;
'REAL' X,Y;
'STRING' S;
'EXTERNAL';
'PROCEDURE' PLOTNI(X,Y,I);
'VALUE' X,Y,I;
'REAL' X,Y;
'INTEGER' I;
'EXTERNAL';
'PROCEDURE' CRSIZE(HEIGHT);
'VALUE' HEIGHT;
'REAL' HEIGHT;
'EXTERNAL';
'PROCEDURE' GRAPHN(XV,YV,M,N);
'VALUE' M,N;
'INTEGER' M,N;
'ARRAY' XV,YV;
'EXTERNAL';
'BOOLEAN' 'PROCEDURE' EVEN(X);
'VALUE' X;
'REAL' X;
'BEGIN'
'INTEGER' Q;
Q:=X;
'IF' ((Q/2)-(Q'/'2))>0,<
'THEN' EVEN:='FALSE'
'ELSE' EVEN:= 'TRUE'
'END';
'PROCEDURE' AXPLOT(X,Y,Z,P);
'VALUE' P,X,Y,Z;
'REAL' X,Y;
'INTEGER' P,Z;
'BEGIN'
'REAL' A,B;
'INTEGER' Q,V;
A:= X;
B:= Y;
POINT(A,B);
'IF'P=1'THEN'JOIN(A*(Z-1)/Z,B)
'ELSE' JOIN(A,B + 0,78/4*(Z-1));
'IF' P=1 'THEN'
'BEGIN' CRSIZE(0,15);
PLOTAS(2,0,9,25,'('%DECAYS')');
PLOTAS(2,0,9,0,'('PER%MINUTE')');
'END'
'ELSE'
'BEGIN' CRSIZE(0,15);
PLOTAS(7,9,0,95,'('R')');
CRSIZE(0,10);
PLOTAS(8,09,0,90,'('M')');
PLOTAS(8,25,0,95,'('X')');
CRSIZE(0,15);
PLOTNI(8,45,0,95,100);
```

```
'END';
'FOR' Q:=1 'STEP' 1 'UNTIL' Z 'DO'
'BEGIN' 'COMMENT' P=1 FOR X AXIS PLOTTING;
POINT(A,B);
'IF' P=1 'THEN' 'GOTO' GL1
'ELSE' 'BEGIN' 'COMMENT' THIS PLOTS THE Y AXIS;
'IF' EVEN(Q)
'THEN' JOIN(A=0,1,B)
'ELSE' 'IF' Q=1 'THEN' JOIN(A=0,2,B)
'ELSE' 'BEGIN' CRSIZE(0,15);
JOIN(A=0,2,B);
V:=500*(Q=1);
PLOTNI(A=0,8,B,V);
'END';
B:=B + 0,7874
'END';
'GOTO' GL2;
GL1: 'BEGIN' 'COMMENT' THIS PLOTS THE X AXIS;
'IF' EVEN(Q) 'THEN' JOIN(A,B=0,1)
'ELSE' 'IF' Q=1 'THEN' JOIN(A,B=0,1)
'ELSE' 'BEGIN' CRSIZE(0,15);
JOIN(A,B=0,1);
V:=10*(Q=1);
PLOTNI(A=0,15,B=0,35,V);
'END';
A:= A + 0,5
'END';
GL2: 'END'
'END';
LIMITS(1,0,9,0,0,0,10,0);
BORDER;
LIMITS(0,0,14.0,0.0,10,0);
AXPLOT(2,0,0,8,11,0);
AXPLOT(2,0,0,8,15,1);
LIMITS(2,0,8,0,0,8,9,4614);
REGION(0,0,120,0,0,0,5500,0);
HISTGM(0,0,0,0,100,0/DYE,DPI,1,N);
LIMITS(0,0,14.0,0.0,10,0);
FRAME;
LIMITS(1,0,9,0,0,0,10,0);
BORDER;
LIMITS(0,0,14.0,0.0,10,0);
AXPLOT(2,0,0,8,11,0);
AXPLOT(2,0,0,8,15,1);
LIMITS(2,0,8,0,0,8,9,4614);
REGION(0,0,120,0,0,0,5500,0);
CURVEO(XPT,DPI,1,N);
GREND;
'END' 'END' 'END'

TNNN
```

REFERENCES

Ackers, G.K. (1964) Biochemistry 3, 723-730.

Andrews, P. (1965) Biochem. J. 96, 595-606.

Anker, H.S. (1970) FEBS Lett. 7, 293.

Benker, G.A. & Cotman, C.W. (1972) J. biol. Chem. 247, 5856-5861.

Banks, P. (1965) Biochem. J. 95, 490-496.

Banks, P. (1966) Biochem. J. 101, 18C-20C.

Banks, P. (1970) Biochem. J. 118, 813-818.

Banks, P., Helle, K.B. & Mayor, D. (1969) Mol. Pharmacol. 5, 210-212.

Banks, P., Mayor, D., Mitchell, M. & Tomlinson, D. (1971) J. Physiol. Lond. 219, 755-761.

Bartlett, G.R. (1959) J. biol. Chem. 234, 466-468.

Bates, R.G. & Bower, V.E. (1956) Analyt. Chem. 28, 1322-1324.

Baumgartner, H., Winkler, H. & Hörtnagl, H. (1973) Eur. J. Pharmacol. 22, 102-104.

Benedeczky, I. & Smith, A.D. (1972) Z. Zellforsch. 124, 367-386.

Berneis, K.H., Da Prada, M. & Pletscher, A. (1970) Biochim. Biophys. Acta 215, 547-549.

Björklund, A., Cegrell, L., Falck, B., Ritzén, M. & Rosengren, E. (1970) Acta physiol. scand. 78, 334-338.

Blaschko, H. (1939) J. Physiol., Lond. 96, 50P-51P.

Blaschko, H., Born, G.V.R., D'Iorio, A. & Eade, N.R. (1956) J. Physiol., Lond. 133, 548-557.

Blaschko, H., Firemark, H., Smith, A.D. & Winkler, H. (1967) Biochem. J. 104, 545-549.

Blaschko, H., Hagen, J.M. & Hagen, P. (1957) J. Physiol., Lond. 139, 316-322.

Blaschko, H. & Helle, K.B. (1963) J. Physiol., Lond. 169, 120P-121P.

Blaschko, H., Smith, A.D. & Winkler, H. (1966) Naunyn-Schmiedebergs Arch. Pharmak. 253, 23.

Blaschko, H. & Welch, A.D. (1953) Naunyn-Schmiedebergs Arch. Pharmak. 219, 17-22.

Bosch, H. van den. & Poorthuis, B.J.H.M. (1972) Communication No. 194 to the FEBS 8th Annual Conference, Amsterdam. North Holland. Amsterdam.

Bretscher, M.S. (1971) Nature New Biology 231, 229-232.

Bretscher, M.S. (1972) J. molec. Biol. 71, 523-528.

Buckley, J.T., Lefebvre, Y.A. & Hawthorne, J.N. (1971) Biochim. Biophys. Acta 239, 517-519.

Carraway, K.L., Kobylka, D. & Triplett, R.B. (1971) Biochim. Biophys. Acta 241, 934-940.

Charlwood, P.A., Pitt-Rivers, R. & Schwartz, H.L. (1970) Biochem. J. 116, 769-772.

Choules, G.L. & Zimm, B.H. (1965) Analyt. Biochem. 13, 336-344.

Chubb, I.W., De Potter, W.P., & De Schaepdryver, A.F. (1972) Life Sci. 11, 323-333.

Clarke, J.T. (1964) Ann. N.Y. Acad. Sci. 121, 428-436.

Coupland, R.E. (1965) The Natural History of the Chromaffin Cell. Longmans. London.

Dahlström, A. (1971) Phil. Trans. Roy. Soc. Lond. B. 261, 325-358.

Dakin, H.D. (1905) Proc. Roy. Soc. Lond. B. 76, 491-497.

De Potter, W.P. (1971) Phil. Trans. Roy. Soc. Lond. B. 261, 313-317.

De Potter, W.P., Chubb, I.W. & De Schaepdryver, A.F. (1972) Arch. int. Pharmacodynam. Ther. Suppl. 196, 258-287.

De Potter, W.P., Smith, A.D. & De Schaepdryver, A.F. (1970) Tissue & Cell 2, 529-546.

De Robertis, E.D.P. & Vaz Ferreira, A. (1957) Exp. Cell Res. 12, 568-574.

De Vito, E. & Santomé, J.A. (1966) Experientia 22, 124-125.

Droz, B. (1969) Int. Rev. Cytol. 25, 363-390.

Dubois, F. B.Sc. thesis, Oxford, 1970.

Duch, D.S., Viveros, O.H. & Kirshner, N. (1968) Biochem. Pharmacol. 17, 255-264.

Dunker, A.K. & Rueckert, R.R. (1969) J. biol. Chem. 244, 5074-5080.

Eade, N. (1957) D. Phil. thesis, Oxford University.

Eagle, H. (1959) Science 130, 432-437.

Edelhoch, H. & Rall, J.E. (1964) in The Thyroid Gland. Ed. by Pitt-Rivers, R. & Trotter, W.R. Butterworths. London. 1, p.113.

Elliot, T.R. (1904) J. Physiol., Lond. 32, 401-467.

Evans, W.H. & Gurd, J.W. (1973) Biochem. J. 133, 189-199.

Euler, U.S. von. (1946) Acta physiol. scand. 12, 73-97.

Fairbanks, G., Steck, T.L. & Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.

Ferguson, K.A. (1964) Metabolism, 13, 985-1002.

Fish, W.W., Mann, K.G. & Tanford, C. (1969) J. biol. Chem. 244, 4989-4994.

Fish, W.W., Reynolds, J.A. & Tanford, C. (1970) J. biol. Chem. 245, 5166-5168.

Flatmark, T., Terland, O. & Helle, K.B. (1971) Biochim. Biophys. Acta 226, 9-19.

Flatmark, T., Lagercrantz, H., Terland, O., Helle, K.B. & Stjärne, L. (1971) Biochim. Biophys. Acta 245, 249-252.

Foldes, A., Jeffrey, P.L., Preston. B.N. & Austin, L. (1973) J. Neurochem. 20, 1431-1442.

Friedman, S. & Kaufman, S. (1965) J. biol. Chem. 240, 4763-4773.

Geffen, L.B. & Livett, B.G. (1971) Physiol. Revs. 51, 98-157.

Geffen, L.B. & Rush, R.A. (1968) J. Neurochem. 15, 925-930.

Gray, R.H. & Steffensen, D.M. (1968) Analyt. Biochem. 24, 44-53.

Greengard, P. & McAfee, D.A. (1972) in Biochem. Soc. Symp. 36, Ed. by Smellie, R.M.S. London: The Biochemical Society. 87-102.

Grynszpan-Winograd, O. (1969) C.R. Acad. Sci. (Paris) 268, 1420-1422.

Hagen, P. & Barrnett, R.J. (1960) in Adrenergic Mechanisms Ed. by Vane, J.R., Wolstenholme, G.E.W. & O'Connor, M. Churchill Ltd. London. 83-99.

Hedrick, J.L. & Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155-164.

Helle, K.B. (1966a) Mol. Pharmacol. 2, 298-310.

Helle, K.B. (1966b) Biochem. J. 100, 6C-7C.

Helle, K.B. (1968) Biochem. J. 109, 43P-44P.

Helle, K.B. (1971) Biochim. Biophys. Acta 245, 80-93.

Helle, K.B., Flatmark, T., Serck-Hanssen, G. & Lönning, S. (1971) Biochim. Biophys. Acta 226, 1-8.

Helle, K.B., Lagercrantz, H. & Stjärne, L. (1971) Acta physiol. scand. 81, 565-567.

Helle, K.B. & Serck-Hanssen, G. (1969) Pharmacol. Res. Commun. 1, 25-29.

Hendry, I.A., Iversen, L.L. & Black, I.B. (1973) J. Neurochem. 20, 1683-1689.

Henle, J. (1865) Z. rat. Med. 24, 143-152.

Hillarp, N.-Å. (1958a) Acta physiol. scand. 42, 144-165.

Hillarp, N.-Å. (1958b) Acta physiol. scand. 42, 321-332.

Hillarp, N.-Å. (1958c) Acta physiol. scand. 43, 82-96.

Hillarp, N.-Å. (1959) Acta physiol. scand. 47, 271-279.

Hillarp, N.-Å., Lagerstedt, S. & Nilson, B. (1953) Acta physiol. scand. 29, 251-263.

Holtz, P., Heise, R. & Lüdtke, K. (1938) Naunyn-Schmiedeberg's Arch. Pharmak. 191, 87-118.

Holzbauer, M. & Sharman, D.F. (1972) in Catecholamines Ed. by Blaschko, H. & Muscholl, E. Handbook of Experimental Pharmacology 33, Springer-Verlag. Berlin, Heidelberg & New York. 110-185.

Hörtnagl, H., Hörtnagl, H. & Winkler, H. (1969) J. Physiol. Lond. 205, 103-114.

Hörtnagl, H., Winkler, H., Schöpf, J.A.L. & Hohenwallner, W. (1971) Biochem. J. 122, 299-304.

Hörtnagl, H., Winkler, H. & Lochs, H. (1972) Biochem. J. 129, 187-195.

Hörtnagl, H., Winkler, H. & Lochs, H. (1973) J. Neurochem. 20, 977-985.

Ichikawa, Y. & Yamano, T. (1965) Biochem. Biophys. Res. Commun. 20, 263-268.

Jacobowitz, D. (1970) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 29, 1929-1944.

Jones, C.T. & Banks, P. (1970a) Biochem. J. 118, 791-800.

Jones, C.T. & Banks, P. (1970b) Biochem. J. 118, 801-812.

Kapadia, G. & Chrambach, A. (1972) Analyt. Biochem. 48, 90-102.

Karlsson, J-O. & Sjöstrand, J. (1971a) J. Neurobiol. 2, 135-143.

Karlsson, J-O. & Sjöstrand, J. (1971b) J. Neurochem. 18, 749-767.

Karlsson, J-O. & Sjöstrand, J. (1971c) J. Neurochem. 18, 2209-2216.

Karlsson, J-O. & Sjöstrand, J. (1971d) FEBS Lett. 16, 329-332.

Kebabian, J.W. & Greengard, P. (1971) Science, N.Y. 174, 1346-1348.

Kirshner, A.G. & Kirshner, N. (1969) Biochim. Biophys. Acta 181, 219-225.

Kirshner, N., Holloway, C., Smith, W.J. & Kirshner, A.G. (1966) in Mechanisms of Release of Biogenic Amines Ed. by von Euler, U.S., Rosell, S. & Uvnäs, B. Pergamon. Oxford. p 109-123.

Kirshner, N. & Kirshner, A.G. (1971) Phil. Trans. Roy. Soc. Lond. B. 261, 279-289.

Klein, R.L. & Lagercrantz, H. (1971) Acta physiol. scand. 83, 70-76.

Koelle, G.B. (1963) in Cholinesterase and Anticholinesterase Agents Handbook of Experimental Pharmacology 15, Ed. by Koelle, G.B. Springer-Verlag, Berlin, Heidelberg & New York. 187-298.

Kohn, A. (1900) Arch. mikr. Anat. 56, 81-148.

Lagercrantz, H. (1971) Acta physiol. scand. suppl. 366, 1-44.

Lagercrantz, H., Klein, R.L. & Stjärne, L. (1970) Life Sci. 9, 639-650.

Langemann, H. (1951) Brit. J. Pharmacol. 6, 318-324.

Larrabee, M.G. & Klingman, J.D. (1962) in Neurochemistry (2nd Ed.) Edited by Elliott, K.A.C., Page, I.H. & Quastel, J.H. Springfield, Ill.: Thomas. p.150.

Larrabee, M.G., Klingman, J.D. & Leicht, W.S. (1963) J. Neurochem. 10, 549-570.

Laverty, R. & Sharman, D.F. (1965) Brit. J. Pharmacol. 24, 538-548.

Libet, B. (1970) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 29, 1945-1956.

Loening, U.E. (1967) Biochem. J. 102, 251-257.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. biol. Chem. 193, 265-275.

Maizel, J.V. jnr. (1966) Science 151, 988-990.

Maizel, J.V. jnr. (1969) in Fundamental Techniques in Virology Edited by Habel, K. & Salzman, N.P. Academic Press. New York. p.334.

Matthews, M.R. & Raisman, G. (1969) J. Anat., Lond. 105, 255-282.

McAfee, D.A., Schorderet, M. & Greengard, P. (1971) Science, N.Y. 171, 1156-1158.

McBride, W.J. & Klingman, J.D. (1972) J. Neurochem. 19, 865-880.

Morris, C.J.O.R., (1967) in Protides of the Biological Fluids 14th colloquium Edited by Peeters, H. Elsevier, New York. p.543.

Mylroie, R. & Koenig, E. (1971) FEBS Lett. 12, 121-124.

Nagatsu, T., Levitt, M. & Udenfriend, S. (1964) J. biol. Chem. 239, 2910-2917.

Neville, D.M. jnr. (1971) J. biol. Chem. 246, 6328-6334.

Oka, M., Ohuchi, T., Yoshida, H. & Imaizumi, R. (1966) Life Sci. 5, 427-432.

Oliver, G. & Schäfer, E.A. (1895) J. Physiol., Lond. 18, 230-276.

Paggi, P. & Toschi, G. (1971) J. Neurobiol. 2, 119-128.

Paus, P.N. (1971) Analyt. Biochem. 42, 372-376.

Peacock, A.C. & Dingman, C.W. (1967) Biochemistry, N.Y. 6, 1818-1827.

Phillips, D.R. & Morrison, M. (1971) Biochemistry 10, 1766-1771.

Poisner, A.M. & Bernstein, J. (1971) J. Pharmacol. Exp. Ther. 177, 102-108.

Poisner, A.M. & Trifaró, J.M. (1967) Mol. Pharmacol. 3, 561-571.

Pusztai, A. & Watt, W.B. (1970) Biochim. Biophys. Acta 213, 463-467.

Redburn, D.A., Poisner, A.M. & Samson, F.E. jnr. (1972) Brain Res. 44, 615-624.

Reithel, F.J. (1963) in Advan. Prot. Chem. Ed. by Anfinsen, C.B., Anson, M.L. & Edsall, J.T. Academic Press. New York and London. 18, 123-226.

Rodbard, D. & Chrambach, A. (1970) Proc. Nat. Acad. Sci. U.S. 65, 970-977.

Rodbard, D. & Chrambach, A. (1971) Analyt. Biochem. 40, 95-134.

Roe, J.H. (1955) J. biol. Chem. 212, 335-343.

Schneider, F.H., Smith, A.D. & Winkler, H. (1967) Brit. J. Pharmacol. 31, 94-104.

Schyns, R. (1968) J. Chromatogr. 36, 549-550.

Segrest, J.P., Jackson, R.L., Andrews, E.P. & Marchesi, V.T. (1971) Biochem. Biophys. Res. Commun. 44, 390-395.

Segrest, J.P., Jackson, R.L., Marchesi, V.T., Guyer, R.B. & Terry, W. (1972) Biochem. Biophys. Res. Commun. 49, 964-969.

Shapiro, A.L., Vinuela, E. & Maizel, J.V. jnr. (1967) Biochem. Biophys. Res. Commun. 28, 815-820.

Smith, A.D. (1968) in The Interaction of Drugs and Subcellular Components in Animal Cells Ed. by Campbell, P.N. Churchill Ltd. London. 239-292.

Smith, A.D. (1972a) Sci. Basis Med. Ann. Revs. 74-102.

Smith, A.D. (1972b) Pharmac. Revs 24, 435-457.

Smith, A.D. (1972c) in Biochem. Soc. Symp. 36, Ed. by Smellie, R.M.S. London. The Biochemical Society. 103-131.

Smith, A.D. (1973) Brit. med. Bull. 29, 123-129.

Smith, A.D. & Winkler, H. (1966) J. Physiol., Lond. 183, 179-188.

Smith, A.D. & Winkler, H. (1967a) Biochem. J. 103, 480-482.

Smith, A.D. & Winkler, H. (1967b) Biochem. J. 103, 483-492.

Smith, A.D. & Winkler, H. (1969) in Lysosomes in Biology and Pathology Ed. by Fell, H. & Dingle, J.T. North Holland. Amsterdam. 1, 155-166.

Smith, A.D. & Winkler, H. (1972) in Catecholamines Ed. by Blaschko, H. & Muscholl, E. Handbook of Experimental Pharmacology 33, Springer-Verlag Berlin, Heidelberg & New York. 538-617.

Smith, W.J., Kirshner, A.G. & Kirshner, N. (1964) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 23, 350.

Smith, W.J. & Kirshner, N. (1967) Mol. Pharmacol. 3, 52-62.

Smith, U., Smith, D.S., Winkler, H. & Ryan, J.W. (1973) Science 179, 79-82.

Sober, H.A., Gutter, F.J., Wyckoff, M.M. & Peterson, E.A. (1956) J. Am. Chem. Soc. 78, 756-763.

Spiro, M.J. & Ball, E.G. (1958) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 17, 314.

Steck, T.L., Fairbanks, G. & Wallach, D.F.H. (1971) Biochemistry 10, 2617-2624.

Steel, R.G.D. & Torrie, J.H. (1960) Principles and Procedures of Statistics McGraw-Hill. New York. p.173.

Stolz, F. (1904) Ber. dtsch. chem. Ges. 37, 4149-4154.

Strieder, N., Ziegler, E., Winkler, H. & Smith, A.D. (1968) Biochem. Pharmacol. 17, 1553-1556.

Takamine, J. (1901) J. Physiol., Lond. 27, xxix-xxx.

Taugner, G. (1971) Naunyn-Schmiedebergs Arch. Pharmak. 270, 392-406.

Taugner, G. (1972) Naunyn-Schmiedebergs Arch. Pharmak. 274, 299-314.

Taugner, G. & Hasselbach, W. (1966) Naunyn-Schmiedebergs Arch. Pharmak. 255, 266-286.

Thoenen, H. (1972) In Biochem. Soc. Symp. 36, Ed. by Smellie, R.M.S. London: The Biochemical Society. 3-15.

Thoenen, H., Kettler, R., Burkard, W. & Saner, A. (1971) Naunyn-Schmiedebergs Arch. Pharmak. 270, 146-160.

Thoenen, H., Saner, A., Kettler, R. & Angeletti, P.U. (1972) Brain Res. 44, 593-602.

Trifaró, J.M. (1972) FEBS Lett. 23, 237-240.

Trifaró, J.M., Collier, B., Lastowecka, A. & Stern, D. (1972) Exptl. Mol. Pathol. 8, 264-267.

Trifaró, J.M. & Dworkind, J. (1970) Analyt. Biochem. 34, 403-412.

Trifaró, J.M. & Warner, M. (1972) Mol. Pharmacol. 8, 159-169.

Triplett, R.B. & Carraway, K.L. (1972) Biochemistry 11, 2897-2903.

Tykva, R. & Votruba, I. (1972) Analyt. Biochem. 50, 18-27.

Udenfriend, S. (1966) Pharmac. Revs 18, 43-51.

Vulpian, A. (1856) C.R. Acad. Sci. (Paris) 43, 663-665.

Warren, L. (1959) J. biol. Chem. 234, 1971-1975.

Weber, K. & Osborn, M. (1969) J. biol. Chem. 244, 4406-4412.

Weinberg, R.A. & Penman, S. (1968) J. molec. Biol. 38, 289-304.

West, G.B. (1955) Quart. Rev. Biol. 30, 116-137.

Winkler, H. (1969) Naunyn-Schmiedebergs Arch. Pharmak. 263, 340-357.

Winkler, H. (1971) Phil. Trans. Roy. Soc. Lond. B. 261, 293-303.

Winkler, H., Hörtnagl, H., Hörtnagl, H. & Smith, A.D. (1970) Biochem. J. 118, 303-310.

Winkler, H., Schöpf, J.A.L., Hörtnagl, H. & Hörtnagl, H. (1972) Naunyn-Schmiedebergs Arch. Pharmak. 273, 43-61.

Winkler, H., Ziegler, E. & Strieder, N. (1966) Nature 211, 982-983.

Wood, J.G. (1967) Amer. J. Anat. 121, 671-704.

Young, R.W. & Fulhorst, H.W. (1965) Analyt. Biochem. 11, 389-391.