# Studies on the Cell Surface and Adhesion of Myoblasts During Early Myogenesis.

A thesis submitted for the degree of Doctor of Philosophy

by

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## Summary.

During myogenesis mononucleated cells, myoblasts, fuse with one another to give rise to multinucleated cells, known as myotubes. This event has been shown to be highly cell specific, in that myoblasts will only fuse with other skeletal muscle cells. It was this specificity that was under investigation. Clearly, cell-cell adhesion and recognition must take place prior to fusion, and it has been suggested that specific adhesion molecule(s) present at the myoblast surface at the time of fusion mediate the specificity of the event.

Four main approaches were undertaken:-

(a) Monitoring transitions in mannose containing glycoproteins as myogenesis procedes.

(b) Affinity chromatography using plasma-membrane proteins coupled to Sepharose.

(c) Comparing the adhesion of 24 hour old (in culture) myoblasts and 48 hour old (in culture) myoblasts to myoblasts and myotubes of varying age.

(d) Raising antibodies against 48 hour old (in culture) myoblasts.

(a) It has been reported that the lectin concanavalin A (Con A) will inhibit the fusion of myoblasts. Therefore, the appearance of a mannose containing moiety at the time of fusion might be involved in mediating fusion. Changes in glycoprotein synthesis were monitored using both one and two-dimensional polyacrylamide gel electrophoresis and iodinated Con A. Numerous complex differences were observed, too many for this approach to be particularly helpful.

(b) A novel approach to affinity chromatography was employed. Plasma-membranes from 48hr old (in culture) myoblasts (fusion competent) were isolated by centrifugation. The proteins were then coupled to Sepharose in the presence of octyl-glucoside. Iodinated cell-surface proteins, also from 48 hour old (in culture) myoblasts, were then added to the column, and allowed to circulate overnight. The column was then washed extensively, and bound proteins were eluted with a salt gradient. This approach should give rise to the isolation of proteins with the ability to bind to each other, either homophilically, or heterophilically. Two peaks were isolated. These were dialysed to a small volume, and prepared for SDS-PAGE. The resulting autoradiograph detected the presence of nine distinct bands, at 250KD, 225KD, 170KD, 140KD, 100KD, 76KD, 58KD, 41KD, and 29KD.

(c) No difference could be detected between the adhesion of 24 hour old myoblasts and 48 hour old myoblasts to myoblasts and myotubes of varying age. The idea behind this approach being that if a developmentally regulated antigen were responsible for the specificity of fusion, and that if its expression were limited to the time of fusion, then one might expect to see an increase in cell-cell and cell-myotube adhesion.

(d) Antibodies to the 48hr old (in culture) myoblasts were raised in a rabbit. IgG was isolated and tested for reactivity. The antibody was then extensively adsorbed against 24 hour old (in culture) myoblasts, until the antibody reacted only with the 48 hour old myoblasts. The 48 hour old myoblasts are fusion competent, whereas the 24hr old ones are

not. An Fab was prepared from this IgG, and tested to see whether it could inhibit cell-cell aggregation, as measured by Coulter counter assay. Results show that while aggregation proceded as normal for 30-45 minutes, the Fab was able to prevent further aggregation, and indeed caused the breakdown of existing aggregates. A non-specific rabbit Fab had no effect on aggregation, as did IgG. This tends to suggest that myoblast adhesion/recognition is at least a two step event.

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List of abbreviations.

| | |
|---|---|
| BDUR | 5-bromo-2-deoxyuridine |
| cAMP | cyclic adenosine monophosphate |
| CHX | cyclohexamide |
| CIG | cold insoluble globulin |
| Con A | concanavalin A |
| csA | contact site A |
| csB | contact site B |
| DEAE | diethylaminoethyl |
| DPBI | Dulbecco's phosphate buffered iodide |
| DPBS | Dulbecco's phosphate buffered saline |
| DPH | 1,6-diphenyl-1,3,5,-hexatriene |
| EDTA | ethylene-diamine tetra acetic acid |
| EGTA | ethylenebis (oxyethylene-nitrilo) tetra acetic acid |
| Fab | antibody fragment |
| IEF | Isoelectric focusing |
| IgG | immunoglobulin G |
| KD | kilodaltons |
| L-CAM | liver cell adhesion molecule |
| LCL | Lens culinaris lectin |
| LETS | large extracellular transformation sensitive |
| LPO | lactoperoxidase |
| N-CAM | neural cell adhesion molecule |
| Ng-CAM | neural-glia cell adhesion molecule |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PE | phosphatidylethanolamine |
| $PGE_1$ | prostaglandin $E_1$ |

| | |
|---|---|
| PHA | phytohemagglutinin |
| $PLA_2$ | phospholipase $A_2$ |
| PLC | phospholipase C |
| PMSF | phenyl methyl sulphonyl fluoride |
| PS | phosphatidylserine |
| $RCA_{120}$ | Ricinus communis agglutinin. |
| SBA | soybean agglutinin |
| SDS | sodium dodecyl sulphate |
| WGA | wheatgerm agglutinin |

# CHAPTER 1:- Introduction.

The fundamental importance of cell adhesion in the formation of tissues and organs during early embryogenesis has rarely been in question. The development from the early embryo to the neonate requires extensive cellular rearrangements, a process that depends upon specific recognition and selective adhesion between cells. The formation of one such tissue, skeletal muscle, has several properties which make it well suited to the study of selective cell adhesion. Not unexpectedly, as myoblasts were amongst the first cells to be grown in dispersed cultures whilst still expressing their differentiated state, the earliest studies were concerned with various aspects of cellular differentiation. Another major line of study has been the process of myoblast fusion.

When histologists first observed adult skeletal muscle tissue in detail they were immediately struck by its unique property of multinuclearity. Each cell, known as a myotube, was elongate and contained many nuclei. Two chief mechanisms were proposed to try and explain this observation. The first, the so-called "amitotic theory", suggested that nuclear division took place without subsequent cell division, thus allowing a build up of nuclei. The second suggested successive fusion of initially mononucleated elements. For many years the amitotic theory was favoured, based almost entirely on one piece of evidence. In fixed sections of skeletal muscle a number of nuclei were "dumbbell" shaped, thus appearing as if they were about to divide. However, with the advent of tissue culture techniques and the pioneering work of Holtzer et al.

(1958) and Cooper and Konigsberg (1961), it soon became evident that mononucleated cells, myoblasts, did indeed fuse to form multinucleated cells, myotubes, and that amitotic division did not occur.

Although the transition from mono- to predominately multinucleated cells can be followed in vivo by taking sections of muscle at various stages in development, it is extremely difficult to carry out any meaningful biochemical analysis of fusion, due mainly to the asynchronicity of the event. Fusion in vivo takes place over a much greater period of time than it does in vitro. In vivo, fusion of myoblasts may still take place after the animal is born (Moss and Leblond, 1970, 1971), with the new myonuclei probably arising from satellite cells (mononucleated cells which lie dormant within the myofibre basal lamina and which are thought to be the reserve pool of myoblasts for muscle regeneration (Mauro, 1961; Reznik, 1970)), although in damaged tissue the new myonuclei may arise from the myonuclei of the damaged myofibres (Walker, 1972; Partridge, 1982)); in vitro, however, using techniques that will be discussed later, it is possible to induce fusion in over 70% of the cells within a few hours.

## Sources of Cells.

The most commonly used source of primary muscle cells has been embryonic chick and quail, isolated by tryptic digestion from either breast or thigh. Other sources of tissue that have been used for primary cells include human (Yasin et al., 1980), calf (Buckingham et al., 1974), mouse (Kawaga et al.,

1977), rat (Yaffe and Feldman, 1964), lizard (Cox, 1968), and even butterfly (Kurtti and Brooks, 1970).

Muscle cell lines exist also, and those most often used were originally isolated from rat muscle, the L6 and L8 lines. The L6 line was established by Yaffe (1968) from embryonic rat muscle who used a protocol designed to "promote multiplication, prevent cell fusion and select myoblasts out of the heterogenous population". Cells from the thigh muscle of new born rats were exposed to 3-methylcholanthrene during the first two passages, grown on feeder layers and repeatedly passaged as soon as the onset of fusion was observed in the cultures. By this method six myogenic cell lines were independently established (Richler and Yaffe, 1970). Of those designated by Richler and Yaffe (1970) L6 has been the most commonly cultured. It is a diploid cell line of high cloning efficiency and almost 100% of the clones will form recognisable muscle fibres if allowed to fuse. Clonal cell lines derived from mouse muscle have also proved popular.

The differentiation that occurs in these cultures is assumed to mimic that which occurs during embryonic development, and most of the information and ideas about the differentiation and fusion of myoblasts during myogenesis come from experimental studies using cultured myoblasts.

## Definition of Fusion.

Fusion *in vitro* can be split into three separate events:- (a) Proliferation, (b) Alignment, adhesion and recognition, and (c) Fusion, that is membrane union.

(a) Proliferation not only involves an increase in cell number, but also allows the cells to become fusion competent. In vitro, just after plating out, the cells undergo an initial burst of division before withdrawing from the cell cycle at the G1 phase (Bischoff and Holtzer, 1968). It has been suggested by Buckley and Konigsberg (1974) that the cells do not actually withdraw from the cell cycle but have an increased G1 phase. They found that before fusion the cell cycle time was short and uniform (10.0 $\pm$ 2.7 hours) becoming greatly increased and more variable in cultures undergoing fusion (19.2 $\pm$ 8.5 hours). Since fusion only takes place in the G1 phase (all nuclei have diploid DNA (Strehler et al., 1963)), they speculate that the probability of fusion increases with the protraction of the G1 phase. However, myoblasts have also been shown to exhibit this withdrawal from the cell division cycle in the absence of fusion (Cox, 1968; Dienstman and Holtzer, 1975, 1977; Adamo et al., 1976).

(b) Alignment, adhesion and recognition are all intimately related. As alignment precedes fusion it is possible that the specificity of fusion shown by myoblasts (see below) is expressed at this stage. During this event the bipolar myoblasts line up along their long axes. It may be that certain cell surface protein(s) unique to myoblasts are involved in a specific cell adhesion which confers the specificity of fusion. Clearly, the cells have to come into contact before they can fuse, and if this intimate contact cannot take place then neither can fusion. It appears, however, that this adhesion is a two stage event. Myoblasts in suspension culture are able to form

"myoballs", the equivalent of myotubes. The strength of adhesion between cells in these aggregates varies with time. Knudsen and Horowitz (1977) grew myoblasts in a $Ca^{2+}$-deficient culture medium for 51 hours (low $Ca^{2+}$ levels prevent fusion from taking place (see later)). These cells were placed in a suspension culture containing 1.6mM $Ca^{2+}$ (fusion permissive conditions). Aggregates that have been allowed to form for 20 minutes can be dispersed easily with the use of ethylene-diamine tetra acetic acid (EDTA), a $Ca^{2+}$ chelater. However, if aggregation is allowed to take place for between 30 and 60 minutes, EDTA is no longer effective, and trypsin is required to disperse the aggregates. Eventually the aggregates become indissociable and by two hours extensive fusion has taken place.

(c) Fusion takes place between the plasma membrane of two adjacent cells, forming a single bilayer that is apparently particle free in freeze fracture replicas (Kalderon and Gilula, 1979). Kalderon and Gilula also noticed the presence of unilamellar vesicles associated with these regions, but which were absent in the myoblast populations whose fusion had been arrested with either 5-bromo-2-deoxyuridine (BDUR), cyclohexamide (CHX), or phospholipase C (PLC). The authors suggest that these vesicles initiate particle depleted membrane domains, giving rise to regions that are enriched with phospholipids. Two particle free regions of plasma membrane of adjacent cells can then fuse to form a single bilayer. The minimum time required for fusion <u>in vitro</u>, from initial contact to nuclear incorporation appears to be in the region of forty

minutes (Capers, 1960). Fusion may occur between cells in end-to-end, end-to-side, or side-to-side configurations (Powell, 1973). Fusion, however, is not a prerequisite for the subsequent biochemical differentiation and accumulation of muscle specific proteins. For example, myosin, creatine kinase, and the acetylcholine receptor, amongst other proteins, will still appear in cells whose fusion has been blocked in a number of ways (Emerson and Beckner, 1975; Moss and Stroham, 1976; Keller and Nameroff, 1974; Turner et al., 1976; Prives and Patterson, 1974).

## Calcium requirement.

As mentioned earlier, the fusion of myoblasts in culture requires the presence of $Ca^{2+}$, although high levels of $Ca^{2+}$ (around $10^{-2}$ M) will inhibit fusion (van der Bosch et al., 1972, 1973). When Shainberg and his co-workers (1969) reduced the $Ca^{2+}$ concentration from 1400uM to 270uM by dialysis, they found that the myoblasts did not fuse. The same result was found by Patterson and Strohman (1972) who simply complexed the ions with the use of ethylenebis (oxyethylene-nitrilo) tetra acetic acid (EGTA). This requirement for $Ca^{2+}$ is specific and all other ions so far tested - $Mg^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Ba^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $La^{2+}$, and $Li^{+}$ - cannot take its place, although it appears that some fusion will take place with $Sr^{2+}$, albeit at the rather high concentration of 2.4mM (Adamo et al., 1976; Schudt et al., 1973). Some of these cations inhibited the effect of $Ca^{2+}$ when they were added simultaneously. In $Ca^{2+}$-deficient medium the early stages of myogenesis proceed as normal.

The cells proliferate and align, but are unable to fuse. When $Ca^{2+}$ is added back to the medium at this stage there is a rapid burst of fusion, with up to 70% of the cells fusing within a few hours (at a rate of more than 15% per hour (van der Bosch et al., 1973)). Myoblasts are able to divide at about the same rate in a low $Ca^{2+}$ medium as they do in normal medium. They also withdraw from the cell cycle at the appropriate time. However, whereas myogenic cells tend to make relatively stable contacts in normal medium, the contacts in low $Ca^{2+}$ are much more transient.

It has generally been assumed that the site of action of $Ca^{2+}$ is at the external surface. Favouring this view is the fact that Bischoff and Lowe (1973, 1974) found that the gentle treatment of myogenic cultures with EGTA and EDTA released substantial amounts of protein from the cells. Attempts have been made to determine whether $Ca^{2+}$ is also acting on cytoplasmic sites. If the intracellular concentration of $Ca^{2+}$ is altered by the use of the calcium ionophore A23187 there is no effect on the fusion rate according to Schudt and Pette (1975). This was later contradicted by David et al., (1981), as they were able to induce "precocious" fusion if A23187 was used only in the 9 hour period prior to fusion. However, $Ca^{2+}$ must enter the cell for fusion to occur as the $Ca^{2+}$-channel blocker D600 will inhibit fusion (David et al., 1981).

So it appears that $Ca^{2+}$ may have more than one function in the process of fusion. Skeletal muscle cells from different species show much the same sensitivity to $Ca^{2+}$. For example, cells from calf, rat, and chick all show about 50% inhibition of

fusion at 500uM $Ca^{2+}$ (Adamo et al, 1976; Shainberg et al., 1969; Schudt et al., 1973; Merlie and Gross, 1976). This requirement for $Ca^{2+}$ has been used as a means of synchronising cell fusion.

Specificity of Fusion.

Isolated skeletal muscle cell tissue usually contains a mixture of at least two types of cell, the myoblast and the fibroblast. Although it is possible to reduce the number of fibroblasts in the population to less than 10% by selective plating (Yaffe, 1968), they still persist. However, these fibroblasts are always excluded from the myotubes that are formed. Using mixtures of myoblasts from different species, where one population has been labelled with [$^3$H]-thymidine before being added to the other, unlabelled population, Yaffe and Feldman (1965) and Yaffe (1969) found that it was possible to obtain hybrid myotubes from calf and rat myoblasts, or rabbit and rat myoblasts. A variety of heterotypic cells such as kidney and liver cells, chondrocytes and dedifferentiated chondrocytes, and smooth and cardiac muscle cells, all labelled with [$^3$H]-thymidine were tested for their ability to fuse with skeletal muscle cells (Okazaki and Holtzer, 1965; Holtzer and Bischoff, 1970; Bischoff, 1978). None of the labelled cells were found to be contained in the resultant myotubes. So it seems that myoblast fusion is cell type specific but not species specific.

Preferential myoblast-myotube adhesion has been shown to take place in vitro. Myoblasts, followed by time lapse

photography, were found to spend around 70% of their time in contact with the myotubes (Bischoff, 1978). If one takes into consideration the fact that the myotubes only occupy some 25% of the field of view, then the myoblasts are in contact with the myotubes two to three times more often than would be expected by random contact. It appears that once the cells make initial contact with the myotubes they go on to to form a fairly stable adhesion. Myotubes are surrounded by a layer rich in glycoproteins and glycosaminoglycans, called the glycocalyx (Bennett, 1963). Loss of this coat by gentle EDTA and EGTA treatment results in the loss of the myoblast's affinity for the myotubes, but does not affect the cell-substratum adhesion, nor the cell's motility.

Studies on the Cell Surface.

Obviously, as was mentioned earlier, this recognition (selective adhesion) must involve the cell surface in some way, and since myoblast fusion is a highly ordered and specific reaction it is reasonable to assume that significant membrane alterations occur to facilitate fusion at the appropriate time. As such, many studies have involved either monitoring for changes in the cell surface or perturbing it by various means and observing the effects on fusion. In general, these studies have been of little value in the understanding of fusion. Although many differences have been described between myoblasts and myotubes, there has rarely been any follow-up work to try and identify a function associated with these

changes. However, as these differences make up a large part of the literature on myogenesis they will be discussed in part.

## The Use of Lectins.

One study that initially looked quite promising arose from the work of Teichberg et al. (1975) who isolated a lectin from the electric organ tissue of the electric eel, Electrophorus electricus. Lectins are proteins that can bind to and cross-link specific carbohydrate groups (Sharon and Lis, 1972). Accordingly, other tissues were investigated for the presence of lectins. Tests for the presence of lectin activity in saline homogenates of tissues and organs from various species revealed "exceptionally high" specific agglutinin titres per milligram of protein in chick muscle, both in vivo and in vitro.

The possibility that this lectin might be involved in myoblast fusion was shown by Gardner and Podleski (1975). Using 15mM thiodigalactoside, they found that they could inhibit the fusion of rat L6 myoblasts. Of the sugars tested, this sugar was found to be the only one effective in blocking fusion, and had no effect on cell proliferation. Gardner and Podleski (1975) thought that a lectin with specificity for beta-D-galactosyl groups mediated the fusion of L6 myoblasts. The idea, that at the time of fusion a lectin was produced that was specific for certain galactosyl groups present only on the surface of myoblasts, could explain the specificity of fusion.

Nowak et al. (1976) not only found lectin activity in L6 cells but also found it to be present in chick embryonic muscle. Both lectins were inhibited with the use of lactose and

thiodigalactoside. The presence of the lectin was found to reach a peak of activity at around the time of fusion. Den et al. (1976) reported similar lectin activity for chick muscle in ovo, but found that thiodigalactoside had no effect on the fusion of cell cultures. The beta-D-galactoside lectin was isolated from acetone powder extracts of embryonic muscle, purified by affinity chromatography and found to be a homo-dimer with a sub-unit molecular weight of 15KD (Den and Malinzak, 1977). It is not clear whether or not this lectin has any involvement in fusion.

It has also been possible to use exogenous lectins as a means of blocking fusion. Den et al. (1975) examined the effect that Concanavalin A (Con A), abrin, and the lectins from wheatgerm (WGA), soybean (SBA) and Lens culinaris (LCL) had on fusion. Using 15ug/ml of each lectin it was found that they inhibited fusion by 62%, 41%, 32%, 8% and 19% respectively. Also by using $^{125}$I-labelled Con A, WGA, and SBA, the number of binding sites was found to be $3.4 \times 10^7$, $6.1 \times 10^7$ and $1.7 \times 10^6$ respectively. Therefore, although myoblasts have twice as many WGA receptors as Con A receptors, Con A is twice as effective at inhibiting fusion as WGA. This suggests that a moiety containing mannose (Con A binds with high affinity to alpha-methyl-D-mannoside) may have an important function in fusion. However, blocking fusion by the use of lectins is rather a clumsy tool, as many lectins, particularly Con A, would be expected to bind to a very wide range of, if not most, glycoproteins (Hughes, 1983). Therefore, the lectins may be simply blocking by steric hindrance.

Continued exposure of the cells to the lectins can prove to be cytotoxic in many cases. This property has led to the isolation of so-called "lectin-resistant variants". These variants, which are unable to bind the lectin and hence survive, usually have defective adhesive properties (Stanley, 1980). Using the lectin Con A, Parfett et al. (1981) and Cates et al. (1984) have isolated muscle cell types that are resistant to its cytotoxic effect. These cell lines have been found to be defective in glycoprotein synthesis and have also lost the ability to fuse, thereby lending further evidence that mannose-containing glycoproteins are involved in myoblast fusion. One particular mutant isolated by Cates et al. (1984), called RI, was found to have a single glycosylation defect. Of all the plasma membrane proteins separated by polyacrylamide gel electrophoresis only one band, with an apparent molecular weight of 46K, failed to bind $^{125}$I-Con A to the same extent as the wild-type. Another mutant isolated by the same authors, called RII, was found to have a reduced capacity to bind $^{125}$I-Con A (about 80% as compared to the wild-type) and was also unable to undergo fusion. However, when a hybrid was made of RI and RII, the mutants complemented each other. Not only does the 46KD glycoprotein reappear, but the ability to fuse is also regained. This tends to suggest that the 46KD protein may have a prominent role in myoblast fusion.

Lectins have also been used to study the gross changes taking place in glycoprotein expression during myogenesis (Burridge and Jordan, 1980). The technique involves separation of the proteins by sodium dodecyl sulphate

polyacrylamide gel electrophoresis (SDS-PAGE). The gel is then immersed in a solution containing the lectin (which is normally labelled with $^{125}I$). Proteins that bind the lectin can then be identified by autoradiography. The glycoproteins of the prefused cells of the rat myoblast cell line G22N and the postfused myotubes have been compared in this way, as well as the non-fusing clone NF2. Various lectins were used:- Con A, WGA, phyto-haemagglutin (PHA), and the lectin from Ricinus communis ($RCA_{120}$). A large number of the bands on the gel react with Con A, more so than the other lectins which tend to bind to just the higher molecular weight components (>80KD). Few changes were noticed between the proteins of the prefused, the fused and the non-fusing cells, although some of the changes were quite prominent. For example, one band, at around 150KD, is prominent in the unfused cultures, but is much reduced in the cultures of the fused myotubes, and in the non-fusing clone; all the lectins labelled a band at 95KD which was present in the myotubes, but present at much reduced levels in the myoblasts and the NF2 cells; WGA labels a band at 110KD which is present on the NF2 cells and on myoblasts, but not on the myotubes. Although these differences could point to glycoproteins involved in the fusion process, this technique does not distinguish them from the appearance of proteins involved in a new differentiated function specific to myotubes.

**Changes in Cell-Surface Protein Expression.**

**Fibronectin.**

The cell surface molecule most studied is probably fibronectin, though most of the work has involved either fibroblasts or sera. Fibronectin, also known as large extracellular transformation sensitive (LETS) protein and cold insoluble globulin (CIG), first attracted attention when it was found to present in greatly reduced amounts on the surface of transformed fibroblasts as compared with normal fibroblasts (Hynes, 1973, 1976; Yamada et al., 1976). Fibronectin has been found not only on the cell surface, but also in the extracellular matrix and in plasma. It is composed of similar polypeptide units of 220-250KD that are linked by disulphide bonds into dimers and higher polymers (Yamada, 1983). The functions of fibronectin appear to be many (Hynes and Yamada, 1982) and are in the main involved with adhesion. It is able to mediate cell attachment and spreading on a number of surfaces. For example, collagen, fibrin, and artificial tissue culture substrates (Culp, 1978; Grinnell, 1978; Grinnell et al., 1980; Kleiman et al., 1981). It also has so-called "binding sites" for heparin, hyaluronic acid and actin (Yamada, 1983). Perhaps surprisingly, the cell-binding region of fibronectin has been shown to very short, consisting of the tripeptide sequence Arg-Gly-Asp (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1985).

Myoblasts require the presence of fibronectin in order to attach and spread upon a gelatin-coated substrate (Ehrisman et al., 1981). It has been shown that myoblasts themselves synthesise very little fibronectin, about one-tenth of the amount skeletal fibroblasts produce (Chiquet et al., 1981).

Despite this, some investigators have studied the changes that take place in the distribution and expression of fibronectin on myoblasts as they proceed from the pre-fusion to the post-fusion state. This has given rise to some apparently contradictory results in the literature.

Hynes et al (1976), using rat L8 cells found that the level of fibronectin increased in post-fusion myotube cultures. Moss et al. (1978) found a similar result with chick myogenic cells, as did Walsh and Phillips (1981), using the mouse G8-1 clonal muscle cell. On the other hand Chen (1977), using rat L8 cells, reported that cell surface fibronectin decreases, accompanied by a change in distribution. Similar results were reported by Walsh et al. (1981) using human muscle cultures and by Furcht et al. (1978) using L6 myoblasts. These differences may be a consequence of the technique used. When cell-surface iodination was used an increase in fibronectin was found; when immunostaining was used there was a decrease.

Gardner and Fambrough (1983) suggested that the differences may be due to abnormal biosynthetic capabilities of myogenic cell lines and that primary cultures contain a mixture of cell types, including fibroblasts which synthesise fibronectin. With the use of monoclonal antibodies they were able to produce pure cultures of both myoblasts and fused myotubes from primary cultures. Ironically, perhaps, they found that there was no difference in the rate of fibronectin synthesis relative to the overall rate of protein synthesis. However, by using an antibody specific for chicken fibronectin, they found that a major alteration in distribution took place between two

and three days in vitro. Prefusion cells were found to have fibronectin present in "discrete, punctate blocks at the abutting surfaces of closely apposed cells", and that this pattern coincides with a period of intense myoblast fusion. Newly formed myotubes, however, had very little fibronectin of their cell surface. The myotubes continued to synthesise fibronectin but released it into the medium, allowing it to attach to the dish. This could explain the differences in expression found by surface iodination and immunostaining.

Whatever the role of fibronectin, it is certainly not involved in the cell-cell adhesion/recognition phase of myogenesis, as cell-cell adhesion and fusion can take place in the absence of both exogenous and endogenous fibronectin (Chiquet et al., 1981). In the presence of high concentrations of fibronectin, fusion is inhibited (Podleski et al., 1979). However myoblasts do display a marked tendency to align themselves along orientated fibrils of purified fibronectin that have been deposited on a substrate. Chiquet et al. (1981) suggest that the formation of myotubes in a certain spatial arrangement during muscle morphogenesis may be regulated by a fibronectin-containing matrix produced by connective tissue cells.

## Use of Lactoperoxidase.

A more general approach to the changes taking place at the cell surface has been taken by a number of authors. Both Cates and Holland (1978) and Moss et al. (1978) used lactoperoxidase-catalysed iodination, a technique that will only label cell surface proteins. (However, it has been reported that

this method doesn't label all the proteins present at the surface (Standring et al., 1978)). Moss et al. (1978), using primary chick cultures, reported quantitative changes in a number of cell surface proteins and little in the way of qualitative change. The most notable change took place in the 245KD region. Prefusion profiles indicated two bands that were present in this region, but as fusion proceeded the two bands were replaced by one. Also as fusion proceeded a band of 10.5KD increased in labelling by almost 100%.

Cates and Holland (1978), also using primary chick cells, focused in on a 70KD protein which showed increased synthesis concomitant with the onset of fusion. (Moss et al. (1978) reported a 72KD cell surface protein that actually decreased during fusion). The authors looked at the effect of 5-bromo-2-deoxyuridine, an inhibitor of differentiation of skeletal muscle cells and hence an inhibitor of fusion, on the protein profiles. They found that the protein profiles closely resembled each other with the exception of the 70KD protein which was present in greatly reduced amounts. The authors suggest that the protein may play a role in fusion.

Lactoperoxidase-catalysed iodination was also used by Walsh and Phillips (1981), in conjunction with the use of the iodinated lectins Con A and WGA (as mentioned earlier) and periodate-tritiated borohydride (a method which labels sialoglycoproteins). This time the mouse G8-1 clonal muscle cell line was used. With lacto-peroxidase-catalysed iodination five cell surface proteins of molecular weight 205KD, 160KD, 70KD, 64KD, and 53KD were revealed that were expressed on

myoblasts only. Four components of 150KD, 140KD, 54KD, and 36KD were found to be expressed on myotubes only. The same four proteins found only on the myotubes were also labelled by periodate-tritiated borohydride treatment.

In contrast Pauw and David (1979), also using lactoperoxidase catalysed iodination of cell surface proteins, but using rat L6 cells, found a number of proteins that appear during myogenesis and a 66KD protein which increases in amount after fusion and myotube formation and then subsequently decreases. Surface proteins of 14KD, 12KD and 11KD behave in a similar manner to the 66KD protein except that their appearance and disappearance were delayed by 24hrs.

A summary of the major changes detailed above is shown in Table 1.

Use of Antisera.

Immunological studies of the cell surface have also been made. In this way, developmental stage-specific cell surface antigens have been found. Friedlander and Fischman (1979), using an antisera raised in rabbits against embryonic chick muscle (anti-M-24), found that the myogenic cells did have antigens that were specific to that cell type, having tested the sera against a variety of cells, including fibroblasts, hepatocytes, cardiac myocytes and brain cells. However, the authors did not show to which antigens the antisera was directed against, nor did they test to see if it had any effect on myoblast adhesion or fusion.

Table 1:- Differences in protein expression between myoblasts and myotubes.

| METHOD | DIFFERENCES | CELL TYPE | AUTHORS |
|---|---|---|---|
| Mutant isolation. | Non-fusing mutant - loss of 46KD protein. | Mutant. | 1. |
| Lectin - gel binding. | 150KD protein prominent in unfused cultures.<br>95KD protein prominent in fused cultures.<br>110KD protein - myoblast specific. | Rat G22N. | 2. |
| LPO | Increased fibronectin on myotubes. | Rat L8. | 3. |
| LPO | ditto. | Chick primary. | 4. |
| LPO | ditto. | Mouse G8-1. | 5. |
| Immunostaining. | Decreased fibronectin on myotubes. | Rat L8. | 6. |
| Immunostaining. | ditto. | Human. | 7. |
| Immunostaining. | ditto. | Rat L6. | 8. |
| Immunostaining. | No change in fibronectin expression. | Chick primary. | 9. |
| LPO | Increase in 10.5KD protein.<br>Doublet at 245KD becomes one band. | Chick primary. | 10. |

| | | | |
|---|---|---|---|
| LPO | Increase in 70KD protein concommitant with fusion. | Chick primary. | 11. |
| LPO | 205KD, 160KD, 70KD, 64KD, and 53KD proteins - which are myoblast specific.<br>150KD, 140KD, 54KD, and 36KD proteins - which are myotube specific. | Mouse G8-1. | 12. |
| LPO | Increase in 66KD protein during myotube formation. | Rat L6. | 13. |

List of authors.

1. Cates et al. (1984).
2. Burridge and Jordan (1980).
3. Hynes et al. (1976).
4. Moss et al. (1978).
5. Walsh and Philips (1981)
6. Chen (1977).
7. Walsh et al. (1981).
8. Furcht et al. (1978)
9. Gardner and Fambrough (1983)
10. Moss et al. (1978)
11. Cates and Holland (1978)
12. Walsh and Philips (1981)
13. Pauw and David (1979).

Lee and Kaufman (1981) raised antisera directed against the myogenic E63 subclone of the ray myoblast line, and used it to distinguish antigenic determinants on the E63 cells and on the non-fusing fu-1 subclone. By indirect immunofluorescence they found the distribution of the antigens to be different. The antisera was then adsorbed onto the fu-1 cells, so that the sera now recognised the E63 cells only. Again this lends further evidence to the suggestion that particular molecule(s) present at the cell surface may mediate fusion. Lee and Kaufman (1981) went on to prepare monoclonal antibodies against the E63 cells. These cells were found to have at least four antigenic determinants that were absent or reduced on differentiated myotubes and that three of these determinants were almost totally absent from the defective non-fusing fu-1 cells. However, all the antigenic determinants investigated were found to be present on rat tissue of non-muscle origin. Again these determinants have not been characterised.

**Possible Involvement of a Metalloendoprotease.**

Of course for "new" antigens to appear on the cell surface protein synthesis is not necessary. Existing proteins may be modified by proteolysis, and this would account for the decreased amounts of the higher molecular weight proteins seen at the cell surface during fusion, and the increased amounts of the lower molecular weight ones (Moss et al., 1978; Pauw and David, 1979). There does seem to be some evidence for this possibility. It is possible that limited proteolysis of the cell surface could reduce steric hindrance or charge restraints

between adjacent cell membranes, or that proteolysis could generate a fusogenic peptide, as happens when certain viruses fuse with their host cells. For example, proteolysis of virus membrane glycoprotein is necessary for fusion of both paramyxovirus (Scheid and Choppin, 1974, 1977) and influenza virus (Bosch et al., 1981; Lazarowitz and Choppin, 1975) with their host cells. Also, limited proteolysis can initiate the fusion of erythrocytes (Lucy, 1984).

Couch and Strittmatter (1983) examined the possibility that something similar happens during myoblast fusion and that protease activity was required for this event. Proteases can be subdivided into four different classes:- carboxyl-, metal-, serine, and thiol- dependent (Barrett, 1977). The authors found that the metalloendoprotease inhibitor 1,10-phenanthroline, which chelates metals such as zinc, prevented the fusion of myoblasts, and that the inhibition was dose dependent. Although 1,10-phenanthroline is a metal chelator, inhibition of fusion was not caused by the chelation of calcium. Serine- and thiol- dependent inhibitors did not prevent myoblast fusion. Pepstatin, a carboxyprotease inhibitor, only delayed fusion, by about 12 hours. The authors suggest that proteolysis by a metalloendoprotease is a necessary step in calcium dependent membrane fusion. Calcium could activate the protease either directly or indirectly through another protein such as calmodulin, or calcium could react with the protein substrate bringing about a conformational change that would make it available for hydrolysis.

Changes in Lipids.

The analysis of changes taking place at the cell surface has not been confined to glycoproteins. The phospholipid, cholesterol, and fatty acid contents have also been examined. However, no changes correlating with the transition from myoblasts, through to fusion competent myoblasts, and onto myotubes were seen (Kent et al., 1974; Boland et al, 1977). Using isolated plamalemmae Kent et al. (1974) could not detect any changes in the lipid : protein ratio, the cholesterol : phospholipid ratio, the phospholipid composition or the fatty acid composition during myogenesis.

The question of whether or not the lipid composition changes during fusion was re-investigated by Wakelam and Pette (1984). They analysed the metabolism of whole cell phospholipids during myoblast fusion, using chick cells whose fusion had been synchronised by growing them in low $Ca^{2+}$. They identified changes in the inositol phospholipids. Phosphatidyl inositol, phosphatidyl inositol-4-phosphate, and phosphatidyl 4,5-biphosphate were metabolised rapidly, with up to 50% being broken down within minutes. This was accompanied by a compensatory increase in the levels of phosphatidic acid and 1,2-diacylglycerol (Wakelam, 1983). Wakelam and Pette (1984) suggest that as the latter two compounds are fusogens they might stimulate fusion. However, given the current thinking on the relationship between the turnover of phospholipids and protein kinase C, and the importance of this pathway as a control mechanism (Berridge, 1984), other interpretations are possible. They also

found that the lipid breakdown was stimulated by the binding of a serum component to a "specific developmentally regulated receptor".

An interesting observation was made when the asymmetry of phospholipids in the plasma membrane of chick and quail myoblasts, chick fibroblasts and of the L6 myoblast was investigated. Sessions and Horowitz (1981, 1983) found that 65% of phosphatidylethanol-amine (PE), and 45% of phosphatidylserine (PS) were externally disposed in the chick and quail myoblasts, compared with 35% of PE and 20% of PS in the chick fibroblast. This represents a 2-3 fold increase in lipids known to be fusogenic in artificial systems. This difference may facilitate membrane union between myoblasts. The L6 myoblast, however, was found to have only 22% of PE and 30% of PS externally disposed. This may account for the much lower rate of fusion of L6 cells when compared to primary myoblasts.

A specific change in the pattern of ganglioside content has been found. Gangliosides are glycolipids with one or more sialic acid residues. Little is known about their function, although it has been reported that one particular ganglioside, $GM_1$, acts as a cell surface receptor for cholera toxin, although obviously this is not the function it was intended for. In L6 myoblasts, the presence of the ganglioside $GD_{1a}$ increases three to four fold during fusion, falling back to prefusion levels after fusion. This increase has not been observed in non-fusing mutants (Whatlet et al., 1976). This ganglioside has been shown to be particularly fusogenic in other systems (Maggio et

al., 1978, 1981). Unfortunately, the changes observed in $GD_{1a}$ have not been shown in primary cultures.

A change in the fluidity of the membrane has also been shown. Prives and Shinitzky (1977) measured the microviscosity of the plamalemma of chick myoblasts using the fluorescence polarisation of 1,6,-diphenyl-1,3,5,-hexatriene (DPH). They found that the microviscosity was halved at the onset of cell fusion and then increased gradually during the post-fusion phase. Similar results were obtained by Herman and Fernandez (1978) using microscopic fluorescence relaxation. It is very difficult to analyse these results quantitatively (Campbell and Dwek, 1984) and it is by no means clear that the probes do not cooperatively perturb membranes. However, these results do seem to reveal changes in the properties of the plasmalemma during the course of fusion and changes in fluidity and microviscosity have generally been assumed to reflect changes in lipid composition or organisation, such as those outlined above.

The possible involvement of membrane lipids has been investigated in two other ways. Enzymes have been used to alter the lipid structure (this is discussed later; see "Use of Enzymes") and various lipids have been added exogenously to see their effect on fusion. It is generally assumed that any effects observed by the addition of lipids are likely to be mediated through fusion proper. However, this is not a reasonable assumption as it has been demonstrated that a number of lipids can alter the adhesiveness of a number of cell types (Curtis et al., 1975, 1979; Hoover et al., 1977).

The addition of lysophophatidylcholine to primary cultures of rat skeletal muscle will inhibit fusion, but only over a narrow concentration range (120-160ug/ml), and at maximum only effected a 20% drop in fusion as compared to control cultures. Cholesterol will also inhibit fusion of cultured myoblasts (van der Bosch et al., 1973). A number of lipids have been investigated by Nakornchai et al. (1981). A number of fatty acids ranging from myrystic to stearic acid, and including saturated, mono-unsaturated and poly-unsaturated acids were able to inhibit fusion. Depending upon the fatty acid employed, up to to 50% inhibition was observed over the range of 20-80ug/ml (high concentrations of fatty acids are toxic). However, the saturated acid arachidonic acid, had little effect and the mono-unsaturated acid, linoleic acid stimulated fusion moderately, which confirmed the observation of Prives and Shinitzky (1977). These findings reflect reports by Boland et al. (1977) that horse serum, which supports a high level of fusion for myoblast cultures, has low concentrations of oleic and palmitic acids (which inhibit fusion at high concentrations) and high concentrations of linoleic acid (which enhances fusion) compared to fetal calf serum. Retinol and glycerol monooleate were also found to inhibit cell fusion. All of these results were found to be reversible.

These results are difficult to interpret in terms of a direct effect on membrane fusion as there is no simple relationship between the structure of the fatty acid and its effect on fusion. In fact, in other systems all of these lipids are fusogens. However, one of the main problems in assessing the

significance of these observations is that all of these lipids were presented to cells in serum-containing media, and serum albumin binds most of them very strongly. So it is not clear what the cells "see" in this experimental system and it is very likely that quite small amounts of these agents are taken up by the cells.

The use of prostaglandin $E_1$ ($PGE_1$) at $10^{-7}$ M is able to induce a burst of "precocious" fusion (Zalin, 1979). This is accompanied by a transient rise in intracellular cAMP. The author suggests a possible role for $PGE_1$ in the regulation of the emergence of fusion competent cells. The rise in intracellular cAMP caused by the action of $PGE_1$ is proposed to lead to the activation of a protein kinase. This in turn leads to the activation or synthesis of cellular components required for myoblast fusion. However, some studies have shown that cAMP inhibits fusion (Wakelam, 1985). In another study the rise in cAMP was found to take place after fusion (Schutzle et al., 1984), suggesting that the rise in cAMP may be a consequence rather than a cause.

## Alterations of the Cell Surface.

### Tunicamycin.

As mentioned earlier, many approaches have involved the modification of the cell surface in some way and observing its effect on fusion. In order to examine the importance of carbohydrate for alignment and fusion Olden et al. (1981) used tunicamycin. Tunicamycin is an antibiotic isolated from Streptomyces lysosuperificus. It inhibits the formation of N-

glycosidic bonds in proteins by blocking the transformation of N-acetylglucosamine-1-phosphate into dolichyl phosphate. The authors found that tunicamycin strongly inhibited fusion of embryonic quail muscle, but had no apparent effect on cell alignment. The inhibition of fusion by tunicamycin could be partly prevented by the use of the protease inhibitor leupeptin and pepstatin. According to Couch and Strittmatter (1983) pepstatin alone causes a delay in fusion anyway, of about 12 hours. Leupeptin, which mainly inhibits thiol- and serine-dependent proteases, has no effect of fusion *per se*. This study provides evidence that myoblast fusion is partially mediated by glycoproteins with asparagine linked oligosaccharides. However, the requirement for the carbohydrate appears to be indirect, in that the carbohydrate portion protects the protein from proteolytic degradation.

Use of Enzymes.

A variety of enzymes are capable of inhibiting fusion. One of the problems inherent with the use of enzymes is that they cause the cells to detach from the dish. Therefore the use of enzymes is limited to those whose action will not affect cell-substratum adhesion, nor affect the motility of the cell. This approach also demands the use of pure enzymes of well-defined specificity and ideally the demonstration that the expected alteration, and only the expected alteration, has taken place.

The use of phospholipase C (PLC), an enzyme which cleaves the polar head from the phospholipids leaving

diacylglycerides, will almost completely inhibit myoblast fusion at the concentration of 0.5ug/ml (Nameroff et al., 1973; Nameroff, 1974). However, as only PLC from Clostridium welchii was effective, but not that from Bacillus cereus, even though the latter enzyme was fully active, it is possible that the results are a consequence of a contaminating molecule. The action of PLC also results in the loss of a cell-surface protein, as measured by $^{125}$I-labelled protein release (Nameroff, 1974). Phospholipase $A_2$ ($PLA_2$) also inhibits fusion. $PLA_2$ produces lysophosphatides by removing the fatty acid from the 2 position of the glycerol. As mentioned earlier (see "Use of Lipids"), addition of exogenous lysolecithin will inhibit fusion (Reporter and Norris, 1973), so it is possible that the mechanism of inhibition by $PLA_2$ is the same as that by the addition of lysolecithin.

Enzymes such as neuraminidase, hyaluronidase, and acid phosphatase are able to inhibit fusion at concentrations many times lower than the concentrations required to cause cell detachment. All of these enzymes reduce the surface charge of the myoblast by removing negatively charged groups. In general, proteases will cause detachment at concentrations required to inhibit fusion, and as such are not of great value in the study of myoblast fusion.

Use of Other Agents.

It is possible to inhibit the fusion of myoblasts with a whole variety of different agents (Holtzer, 1970). The problem arises, however, as to whether the agent used is having an

effect on fusion proper, or whether it is merely preventing the emergence of fusion competent cells. In general, any compound that interferes with the proliferation of myoblasts will have an effect on fusion. If the cells are allowed to reach fusion competence, but are prevented from fusing by low $Ca^{2+}$ then the inhibitor could be added along with the $Ca^{2+}$. In this way it would be possible to say whether or not the inhibitor has an effect on fusion proper.

In a set of experiments meeting these requirements it was shown that fusion was an energy requiring process (Bischoff, 1978). The use of azide, cyanide, fluoride, and dinitrophenol (all metabolic poisons) in the absence of glucose, resulted in the inhibition of fusion (Bischoff, 1978). This block was reversible, as normal fusion occured if the inhibitors were removed. However, it appears that the $Ca^{2+}$-induced fusion does not require either RNA or protein synthesis. Cyclohexamide, actinomycin D (Adamo et al., 1976) or 5-fluorouridine (Easton and Reich, 1972) were all unable to block fusion.

## Cell Adhesion.

As yet a cell adhesion molecule for muscle cells has not been identified (although an Fab' has been raised by Turner and Gibralter (1985) that inhibits myoblast aggregation - see Chapter 5), but at this point it is pertinent to discuss tissues and other systems where putative cell adhesion molecules have been found, the techniques used to identify them, and the very nature of adhesion itself. Perhaps surprisingly, despite all the

effort, the biochemical nature of cell adhesion is still poorly understood. In the animal as a whole, the different cell types present display different properties. Some cells are firmly bound together, such as various epithelial cells, while others, such as erythrocytes are freely circulating. Cells, such as neutrophils, can alter their adhesive state in response to infection. Yet even the adhesive properties of these cells can change. Epithelial cells can give rise to tumours, whose cells are particularly invasive and can move outwith their normal constraints. Erythrocytes that have become old and damaged are removed from the circulation by binding to hepatocytes.

Undoubtedly these changes in behaviour reflect a change at the cell surface. In the main two theoretical models have been applied to the study and analysis of adhesion:-

(a) The first suggests that adhesion is the result of long-range electrostatic forces or van der Waals interactions (Curtis, 1967). For example, van Oss et al. (1975) have likened cell-cell adhesion to the hydrophobic interaction seen between colloidal particles.

(b) The second suggests that specific effector molecules present at the cell surface mediate adhesion, whether through direct interaction or bridged by a ligand.

Clearly eletrostatic and electrodynamic interactions between charged molecules are inescapable and must contribute to adhesion in some way (Curtis, 1960, 1962). However, their contribution may be small (Edwards, 1983). Evidence for the existence of specific effector molecules has been obtained from the use of antibodies that are able to

inhibit adhesion. It is important to point out that monovalent antibodies (Fab') are used, otherwise cross-linking of cell surface antigens would occur, and indeed would also lead to the linking of cells together, thereby masking the cells' own adhesion mechanism. This tool has led to the discovery of a number of molecules thought to be involved in adhesion. Two of the better known systems will be discussed : the slime mould and the neural cell adhesion molecule.

## The Slime Mould.

The slime mould most studied is Dictyostelium discoideum. It lends itself particualrly well for study due to its simple lifestyle and ease of manipulation. During times of plentiful food supply Dictyostelium exists as a free-living amoebae and multiplies asexually. However, in response to starvation it forms an aggregate under cyclic-AMP-mediated chemotaxis. This leads to the formation of a fruiting body. Spores are released and the whole cycle starts over again.

The fact that the cell surface antigens of Dictyostelium change during their developmental cycle was first shown by the agglutination studies of Gregg (1956, 1960) and Gregg and Trygstad (1958). Using antisera directed against aggregating cells, they showed that agglutinability increases during the change from the growth phase to the aggregation phase, thus showing the presence of an antigen peculiar to the aggregating cells. Complement fixation has also been used to show the appearance of new cell surface antigens (Sonneborn et al., 1963). Changes that take place at the cell surface have also

been followed by lactoperoxidase-catalysed iodination, followed by SDS-PAGE. Sui et al. (1976) found a total of nine polypeptides in which changes took place during development. Following the cell surface changes for their ability to bind Con A, West and McMahon (1977) found twelve glycoproteins which increased in terms of Con A binding activity between growth phase and aggregation competent phase. In an attempt to identify the particular molecule(s) involved in the adhesion of aggregating cells, Gerisch and his co-workers employed the following strategy:- to raise antisera against cell membrane antigens, to test the ability of Fab' prepared from this sera to block adhesion of aggregating cells and to purify the components that neutralise the ability of Fab' to prevent adhesion (Beug et al., 1970, 1973; Huegson and Gerisch, 1975; Muller and Gerisch, 1978; Gerisch, 1980).

The antigens to which the Fab' binds are then thought to be candidates for molecules that mediate cell-cell adhesion. Curtis (1986) has thrown some doubt onto the validity of such thinking. Since Fab' molecules are of appreciable size (they can be considered to be ellipsoids of 6nm in length and 4nm in diameter) and have one reactive site through which they bind to the cell surface, it could cover the cell with what is essentially a non-adhesive coat. By introducing an artificial antigen onto the cell surface, and by using an Fab' directed against this antigen, Curtis (1986) was able to inhibit the adhesion of cells. Gerisch (1986) acknowledges that because the Fab' is so large it is not of great value as a probe for the substructures of the cell surface molecules whose interaction is

believed by many to be responsible for cell-cell adhesion. Once these candidates have been identified it is necessary to show that they have an actual function in mediating cell-cell adhesion. This can be done in one of two ways:-

(a) Incorporating the purified Fab'-binding protein into liposomes (Hoffman and Edelman, 1983), although this method is open to interpretation, as the protein may not interact in its normal way, and

(b) Selecting mutants that have defective synthesis of the Fab'-binding proteins (both the protein backbone and the carbohydrate residues (Gerisch, 1986)).

The latter strategy was adopted for Dictyostelium discoideum.

In common with other cell adhesion systems, Dictyostelium discoideum displays two different adhesion mechanisms, one EDTA-stable and the other EDTA-labile. (Mouse embryonic and teratocarcinoma cells can simultaneously operate two independent types of intercellular adhesion, a $Ca^{2+}$-dependent and a $Ca^{2+}$-independent one (Takeichi et al., 1979)). It was found that the growth phase cells of D. discoideum could form only the EDTA-sensitive contacts whereas the aggregation-competent cells could form EDTA-stable as well as EDTA-sensitive contacts (Gerisch, 1961). These contacts can be blocked independently by Fab' of appropriate specificity (Beug et al., 1973). Two distinct classes of contact site were discovered. The first, contact site A (csA), was found to be restricted to aggregation competent cells and was responsible for the end-end adhesion that took place when

the cells were forming streams while undergoing aggregation. This contact was EDTA-resistant. The second, contact site B (csB) was found to be common to both growth phase and aggregation competent cells. This contact was EDTA-sensitive.

From the membranes of aggregation competent cells an 80KD glycoprotein has been isolated, which is thought to be responsible for the csA activity (Muller et al., 1979). This glycoprotein is specifically expressed in aggregation competent cells and is able to neutralise the Fab' species that block the EDTA-stable adhesion (Muller and Gerisch, 1978). A glycoprotein of 126KD has been identified in growth phase cells as a target site for the Fab' that blocks the EDTA-labile adhesion (Chadwick and Garrod, 1983).

Gerisch (1986) has pointed out the similarities between the chemical nature of csA from D. discoideum and the neural cell adhesion molecule (N-CAM) from chicken embryos (N-CAM will be discussed in further detail in the following section). Both proteins are integral membrane proteins; both have negatively charged carbohydrates; both are further modified by acylation and phosphorylation. CsA is the most prominently sulphated protein of aggregating cells (Stadler et al., 1983), and while N-CAM is also sulphated (Sorkin et al., 1984) it is also polysialyated (Rothbard et al., 1982). The protein backbone of csA is phosphorylated at serine residues (Schmidt and Loomis, 1982) and that of N-CAM is phosphorylated at threonine residues in addition to serine residues (Sorkin et al., 1984).

While none of the modifications mentioned is unique to cell adhesion molecules, the fact that their combination is

present in contact sites from unrelated organisms may indicate that they are important in the function of these cell adhesion molecules.

Neural Cell Adhesion Molecule (N-CAM).

The use of inhibitory Fab' has also been employed by Brackenbury et al. (1977) to investigate the mechanism of adhesion between cells of the 10-day chick embryonic neural retina. They raised antibodies against chick retinal cells in a rabbit, and found the resulting Fab' from this polyspecific sera inhibited aggregation dramatically. In order to identify the antigens involved, Thiery et al. (1977) tested substances released into the culture medium by retinal tissues for their ability to inhibit aggregation of trypsinised cells. This gave rise to three polypeptides which had molecular weights in SDS of 140KD, 120KD and 65KD. These polypeptides were further purified and antibodies raised against them. Immunoprecipitation of a detergent extract of retinal cell membranes by these antibodies yielded a polypeptide which had a molecular weight of 140KD in SDS. This protein is known as neural cell adhesion molecule (N-CAM). Later, chicken N-CAM was found to be composed of proteins of molecular weight 170KD and 140KD that arise by alternate splicing of mRNA from a single N-CAM gene (Murray et al., 1986). Antibodies against N-CAM have been used to examine both its distribution on retinal cells and to identify other cells that express N-CAM on their surface.

The distribution of N-CAM on the surface of aggregated retinal cells in vitro was not confined to areas of cell-cell contact, but appeared to be evenly distributed. A variety of cells including embryonic muscle cells also have N-CAM on their surface (Grumet et al., 1982), and anti-N-CAM inhibits heterologous adhesion between nerve and muscle cells (Rutishauser et al., 1983).

N-CAM has been isolated in sufficient quantities to carry out extensive biochemical analysis. (By 1985 Edelman and his co-workers had used one-half million embryos to obtain milligram quantities of N-CAM (Edelman, 1985)). N-CAM is particularly rich in sialic acid, with, on average, 130-150 residues of sialic acid per molecule. During the perinatal period N-CAM is modified, leading to the loss of about two-thirds of its polysialic acid. This is known as the embryo to adult, or E-A, conversion. N-CAM binds directly to the N-CAM of an opposing cell, that is homophilic binding (Hoffman and Edelman, 1983). Purified N-CAM has been inserted into artificial lipid vesicles (Rutishauser et al., 1982). These vesicles were able to adhere to cells from the retina, brain and muscle (all of which express N-CAM on their surface), but not to hepatocytes, trypsinised retinal cells or fibroblasts (cells which do not express N-CAM).

Other cell adhesion molecules that have been identified by this method include Ng-CAM (neural-glia) and L-CAM (liver). The use of Fab' has also led to the identification of a protein called uvomorulin, which is thought to be responsible for the compaction of cleavage stage mouse embryos (Hyafil et al., 1980). Uvomorulin is now thought to be identical to L-CAM.

From the literature it appears that different types of molecule are capable of mediating a specific cell interaction. For example, in the marine sponge Microciona prolifera the aggregation factor is a proteoglycan-like molecule with a molecular weight of 2 x $10^7$ daltons (Burger and Misevic, 1986); an 8,500 dalton cell surface glycoprotein has been isolated which is believed to mediate the adhesion of chick limb bud cells (Bee and von der Mark, 1986). At present the number of known mammalian cell adhesion molecules is small. Edelman (1983) has suggested that adhesion processes are mediated by the expression and modulation of only a few adhesive molecules. It has also been suggested that adhesion may involve many different molecules and that while each of them makes a contribution towards adhesion, no particular one is uniquely important (Curtis, 1986). Whatever the case, the answer to specific cell-cell adhesion, such as that displayed by skeletal myoblasts, is sure to lie at the cell surface.

# CHAPTER 2 :- Materials and Methods.

1. Cell Culture.

Muscle cell cultures were obtained from the hindlimbs of 12-day old embryonic White Leghorn chickens. The upper part of the hindlimb was skinned and boned, and the remaining tissue was subjected to versene (BDH) : trypsin (Difco.) digestion (4:1) (versene = 0.55mM EDTA (Ethylene diamine tetra acetic acid) in phosphate buffered saline, pH adjusted to 7.4; trypsin = 0.25% w/v in tris-saline (pH 7.4)) for 30 minutes at 37°C. The tissue was dissociated using a Pasteur pipette, before being passed through a 100um Nitex filter, followed by a 10um Nitex filter to produce a single cell suspension. The cell suspension at this stage contains both fibroblasts and myoblasts. In order to reduce the number of fibroblasts present, the cells are placed in a tissue culture flask in the presence of growth medium at 37°C for 40 minutes. The fibroblasts will adhere to the tissue culture plastic, but the myoblasts will not. Therefore the medium, when it is poured off, contains a reduced number of fibroblasts. The myoblasts are then plated out on gelatin coated tissue culture plastic according to the required needs. The growth medium, Hams F10 (Flow), is supplemented with 5% embryonic extract (Flow) and 15% horse serum (Flow).

2. Collagen Isolation and Gelling.

Type I collagen was prepared from rat tail collagen as described by Schor (1980). Tendons were stripped and solubilised in 3% v/v acetic acid for two days at 4°C. Insoluble material was removed by centrifugation at 3000g for 30 minutes and the clear tropocollagen solution mixed with an

equal volume of 20% w/v NaCl to precipitate the collagen. The collagen was pelleted by centrifugation at 3000g for 45 minutes, washed twice in distilled water, and resuspended in 3% v/v acetic acid at a final concentration of 3mg/ml as determined by $OD_{230}$ compared with a calibration curve prepared from standard solutions of freeze-dried sample of the collagen preparation. The collagen was then dialysed exhaustively against distilled water and adjusted to pH4.0 with HCl.

Collagen gels were reconstituted from rat tail collagen (Type I) as described by Elsdale and Bard (1972). 5ml of the collagen stock solution (3mg/ml in distilled water, adjusted to pH4.0) was mixed at 4°C with 0.5ml of HAMS F10 and 50ul of 0.142M NaOH to restore physiological pH and ionic strength. This was then diluted with growth medium (without serum) to the desired concentration (usually 1mg/ml) and 1ml aliquots were placed in tissue culture grade chambers (10mm diameter). The collagen polymerised in approximately 15 minutes at room temperature, although the gels were incubated for 2 hours at 37°C in a humid atmosphere before use as this prevented detachment of the gels from the wells on addition of medium.

3. Iodination procedures.

Two distinct iodination procedures were carried out:-

(a) The iodination of purified Con A (Sigma), and

(b) Cell surface iodination of living cells.

Both procedures followed that of Markwell and Fox (1978) using the compound 1,3,4,6-tetrachloro-3,alpha-,6,alpha-diphenylglyco-uril, also known as Iodogen (Pierce).

(a) A small amount of Iodogen, dissolved in chloroform, was added to a glass test tube and evaporated to dryness under a stream of air, to leave a coat of Iodogen (100ug) around the bottom of the tube. 10mg of Con A in 0.5ml PBS, containing 1mM alpha-methyl mannoside (Sigma), was added to the reaction vessel. Iodination starts immediately upon the addition of $Na^{125}I$ (500uCi). This mixture was left for 15 minutes at room temperature, being slightly agitated every 5 minutes. The reaction stops as soon as the liquid is removed from the reaction vessel. The free iodide was removed from the labelled Con A by gel filtration (using a Bio-gel P10 disposable column (Bio-Rad)).

(b) Cells to be cell surface labelled were grown on 10cm plastic dishes. The dishes were washed three times with 5ml of PBS (containing 5mM glucose). Two glass coverslips, each previously coated with 100ug of iodogen, were floated on each dish. 500uCi $Na^{125}I$ were added to each dish, and gently agitated by shaking every few minutes. 15 minutes after the addition of the $^{125}I$ the coverslips were removed and the dishes were washed 10 times with 5ml aliquots of DPBI followed by 5ml of DPBS. The cells were then harvested accordingly.

4. Lawn collection assay.

1ml of rat tail collagen (1mg/ml) was placed in 1cm tissue culture wells and allowed to gel. Each well was then washed twice with 1ml growth medium before $10^5$ cells in 1ml

growth medium were added on top of the gels. After 48hrs, $10^{-5}$M cytosine arabinoside (Sigma) was added to each well to prevent the proliferation of fibroblasts. This gave rise to reasonably pure cultures of myotubes (see Chapter 4). Myoblasts were also grown in 25cm$^2$ tissue flasks at a density of $10^6$ cells/ml in 5ml of growth medium. These cells were labelled with $^{51}$Cr, simply by adding 250uCi of Na$^{51}$Cr to the growth medium for at least 8hrs prior to use. After either 24hrs or 48hrs (since plating out) the cells were removed from the flask by mild trypsin:versene treatment, and washed twice in cold growth medium (4°C). $10^5$ labelled cells were added to each well, and the plate was placed in an incubator at 37°C for 50mins. After this time the wells were gently washed three times with 1ml growth medium to remove unattached cells. 1ml of ammonium hydroxide is then added to each well and the $^{51}$Cr content counted in a gamma counter (WilJ 2000).

5. SDS-PAGE, IEF and 2D-PAGE.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis reagents and techniques used were as decribed by Laemmli (1970). Isoelectric focusing and two-dimensional electrophoresis of proteins were carried out according to O'Farrell (1975). The pH gradients had a range from pH 3-10 and % acrylamide was normally 10%.

6. Autoradiography.

(a) Labelling SDS-PAGE gels with $^{125}$I-Con A.

Once completely destained the gel was placed in Buffer "A" for 5-6hrs. The gel was then immersed in a solution of

Buffer "A" containing $^{125}$I-Con A with 0.01% thimerosal (Sigma) overnight. On removal from the solution the gel was washed extensively for 2 days with 3-4 changes of Buffer "A" each day. The gel was dried and exposed to Kodak X-Omat H film with a Cronex hi-plus intensifying screen at -70°C for 3-4hrs before being developed.

(b) SDS-PAGE gels with labelled proteins were dried and exposed to film as described above.

7.Antibody production, isolation, adsorption, and Fab.

(a) Preparation of antiserum.

The protocol was similar to that of Friedlander and Fischman (1979), but varied in the preparation of the antigenic material. Freshly dissociated single muscle cells were plated out on 10cm tissue culture dishes at a density of $10^6$ cells/ml in 10ml of growth medium, and incubated at 37°C for 48hrs. After this time the dish was washed three times with 10ml of ice-cold Hanks Hepes. The cells were scraped from the dish with the use of a rubber policeman in 10ml of ice-cold Hanks Hepes. The clumps of cells were dispersed with the use of a pasteur pipette and centrifuged at 2,000 r.p.m. and 4°C. The cells were resuspended in 10ml of Hanks Hepes and centrifuged three times. The cells were finally suspended in 1ml of Hanks Hepes. This was emulsified with an equal volume of complete Freund's adjuvant (Welcome). The primary injection was administered via multiple portal routes and included intramuscular, subcutaneous, intradermal and intraperitoneal sites. The rabbit was injected with identically prepared cells in 2ml of Hanks Hepes (without Freund's

adjuvant) via the marginal ear vain two weeks later. (Booster injections were administered as required). The cells were always freshly prepared prior to the injections. The rabbit was bled via a nick in the ear on alternate days, 7-15 days after the final injection. The immune serum was separated from the whole blood after allowing clot retraction to take place overnight at 4°C. All serum was heat-inactivated by heating in a water bath at 56°C for 30mins and was then sterilised by passing it through a 0.22um filter (Millipore). Immune serum was stored at either 4°C or -20°C.

(b) Isolation of IgG.

IgG was isolated from immune serum by use of ion exchange chromatography (Fahey and Terry, 1973). Immune serum was dialysed against 0.005M phosphate buffer, pH 8.0, before being added to a diethylaminoethyl-cellulose (DEAE-cellulose (Whatman)) column. Under these conditions IgG was found to pass straight through the column. The activity of IgG was tested by means of a $^{51}Cr$-release cytotoxicity assay (see Section 8).

(c) Adsorption of antisera.

Adsorption of antisera was carried out on monolayers of 24hr old (in culture) myoblasts. 5ml of immune sera were added to 15ml of Hanks Hepes and added to an $80cm^2$ flask of myoblasts, originally plated out at a density of $10^6$ cells/ml in 20ml of growth medium and left for 4hr. The growth medium was removed and the immune sera was added to the flask. The flask was placed on a shaking table and incubated at 37°C for 24hr. The Hanks Hepes/serum mixture was removed and centrifuged at 2,000 r.p.m. for 5 minutes. The supernatant was

then passed through a 0.22um filter and force-dialysed back to its original volume. The antisera was tested for its reactivity against 24hr and 48hr old (in culture) myoblasts by $^{51}Cr$-release cytotoxicity assay. The adsorption process needed to be repeated several times before most of the reactivity against 24hr old myoblasts disappeared.

(d) Preparation and isolation of Fab.

The method was as employed by Porter (1959) and as outlined by Stanworth and Turner (1973). 10mg of IgG was incubated at 37°C for 16hr in the presence of 0.1mg of mercuripapain (Sigma) in 0.1M phosphate buffer, pH7.0, 0.01M cysteine (Sigma), and 0.002M EDTA (to activate the enzyme). The resultant digest was then dialysed extensively against several changes of distilled water over the next 48hr. This was then further dialysed against a 0.1M sodium acetate buffer (pH 5.5). After the final dialysis the sample was placed on a carboxymethyl-cellulose (Whatman) column. After collecting 40ml of 0.1M sodium acetate (pH 5.5) from the column, a gradient of 0.1-0.9 M sodium acetate (pH 5.5, 100ml total) was passed over the column, using a mixing chamber, to elute the bound protein. The fractions were tested by UV absorption (at 280nm) to find those containing protein. The profile indicated three peaks. Peaks I and II were those containing the Fab fragments. However to ensure purity, only the first peak was used in any experimental work. The fractions giving rise to the first peak were dialysed to a small volume (1ml), split into two aliquots, and stored at 4°C and -20°C before use.

8. $^{51}$Cr-release cytotoxicity assay.

Myoblasts were plated out in 96 well microtitre plates at a density of $10^4$ cells/well in 0.1ml of growth medium. To label the cells 1.0 uCi of Na $^{51}CrO_4$ in 0.1ml of growth medium was added to each well and left to incubate at 37°C overnight. Excess $^{51}$Cr was removed from the plates by washing 3 times with Hanks Hepes, and then the plates were reincubated for a further 4hr in growth medium. The cultures were then washed twice more. To test the cytotoxicity of the antisera, 0.05ml of the antiserum and 0.05ml of guinea pig complement (final dilution of 1/10) were added to each well and incubated at 37°C for 45 minutes. After this time 0.1ml of cold Hanks Hepes was added to each well. The liquid contents of each well were carefully removed and each well was then washed out twice with 0.1ml of Hanks Hepes. The liquid collected from each well was then measured for $^{51}$Cr radioactivity (by use of a WilJ 2000 gamma counter). Due to the fact that when $^{51}$Cr enters a cell it is altered in such a way that it is now complexed to proteins within the cell, any radioactivity found in the medium indicates a loss of membrane integrity of that cell (i.e. lysis). Percent cytotoxicity was calculated by the following formula:-

$$\frac{(\text{(}^{51}\text{Cr released in antisera + complement) - background)}}{\text{(}^{51}\text{Cr released in Triton X-100) - background)}} \times 100$$

(Background counts were those released by complement alone, and Triton X-100 (Sigma) was used at a concentration of 1%).

9. Coulter counter assay of cell aggregation.

25ml conical flasks were siliconised to prevent cells from sticking to the sides of the flask. $10^5$ cells in 5ml of growth medium were placed in each flask along with either IgG, MFab or SFab (see Chapter 5 for further details). The flasks were placed on a shaking table within a water bath and incubated at 37°C. 0.1ml samples were taken at the appropriate time interval, placed in 20ml of a saline solution and counted in a Coulter counter.

10. Membrane isolation.

Myoblast plasma membranes were isolated by the method of Cates and Holland (1978) with some minor modifications. Forty eight hours after plating out (at an original density of 1.2 x $10^7$cells/10cm dish), 20 such dishes (one of which was cell surface labelled with $^{125}I$ - see section 3) were washed twice with cold PBS and cells were collected by scraping with a rubber policeman in 3ml of PBS (containing 0.2mM PMSF (Sigma)) per dish. The cells were then centrifuged for 5 minutes at 2,000 r.p.m. and 4°C. The pellet was suspended in 0.25M sucrose (containing 0.2mM PMSF)/1mM triethylanolamine hydrochloride, pH 7.4 (15ml/g wet weight of cells) and briefly homogenised (15 strokes of the pestle). The homogenate was centrifuged for 10 minutes at 2,000 r.p.m. and 4°C. The supernatant was collected and the pellet resuspended in half the original volume of 0.25M sucrose/1mM triethanolamine hydrochloride (containing 0.2mM PMSF) and centrifuged for another 10 minutes at 2,000 r.p.m. and 4°C. Both supernatants were pooled and centrifuged

for 1hr at 20,000 r.p.m. and 4°C. The pellet was resuspended in 1ml of 0.25M sucrose/1mM triethanolamine hydrochloride (containing 0.2mM PMSF), pH 7.4, and layered onto a discontinuous sucrose gradient which was as follows : 0.5ml of 55% (w/w) sucrose, followed by 2ml each of 40%, 32%, 27%, and 20% (w/w) sucrose. The gradients were formed in 10ml capacity tubes and centrifuged for 1.5hr at 53,000 r.p.m. and 4°C. Fractions were collected at each of the interfaces as follows : fraction I, 20% (w/w) sucrose; II, 20-27% (w/w) sucrose; III, 27-32% (w/w) sucrose; IV, 32-40% (w/w) sucrose; V, 40-55% (w/w) sucrose. The fractions were diluted 10-fold with 1mM triethanolamine hydrochloride (containing 0.2mM PMSF), pH 7.4, and centrifuged for 40 minutes at 40,000 r.p.m. and 4°C. The pellets were resuspended in 0.1ml of 1mM triethanolamine hydrochloride (containing 0.2mM PMSF), pH 7.4, and tested for radioactivity ($^{125}I$). Most activity was obtained in fractions II and III. These fractions were pooled. Membranes from six such isolation procedures were collected.

11. Affinity chromatography.

Isolated plasma membrane proteins were coupled to CnBr- activated sepharose using the procedure outlined in "Affinity Chromatography : Principles and Methods" (Pharmacia Fine Chemicals), but with some minor modifications. Six grammes of CnBr-activated Sepharose 4B (Sigma) were washed with 1mM HCl (200ml/g) and then swollen in PBS. One ml of PBS containing 1% octyl glucoside (Sigma) was added to 2mg of the plasma membrane protein and left for one hour at 4°C. After this time the protein was dissolved in 5ml of coupling

buffer ($NaHCO_3$ buffer (0.1M, pH 8.3)) containing 0.5M NaCl. The solution was added to the gel suspension and gently mixed in an end-over-end mixer for 24hr at 4°C. Normally 5-10mg of protein are added per ml of gel. In this case the ratio was 1mg of protein per 10ml of gel. The protein-Sepharose was then washed with coupling buffer and placed in an ethanolamine solution overnight. This washes away excess protein and blocks any active groups remaining. This is followed by a wash in acetate buffer (0.1M, pH 4 containing NaCl (0.5M) followed finally by another wash in coupling buffer. The plasma membrane -Sepharose conjugate was then packed into a column and washed with 5 bed volumes (100ml) of 0.1mM glycine in PBS. The column was then further washed with 5 bed volumes of PBS containing 0.1% Sodium Azide. The column was now ready to use.

Ten dishes of cells (10cm dishes, originally plated out at 1.2 x $10^7$cells/dish and left for 48hr at 37°C) were cell surface iodinated (see iodination procedures). The iodinated cells were solubilised in PBS containing 0.1% octyl glucoside and 0.2mM PMSF at 4°C overnight. The sample was then added to the column and allowed to circulate overnight (all at 4°C). Unbound iodinated proteins were then washed from the column with PBS (fractions were collected and tested for radioactivity). The bound proteins were eluted with a linear NaCl gradient (0-1M) in 100ml. 2ml fractions were collected and tested for radioactivity. Those fractions that contained radioactivity were force dialysed to a small volume and prepared for SDS-PAGE.

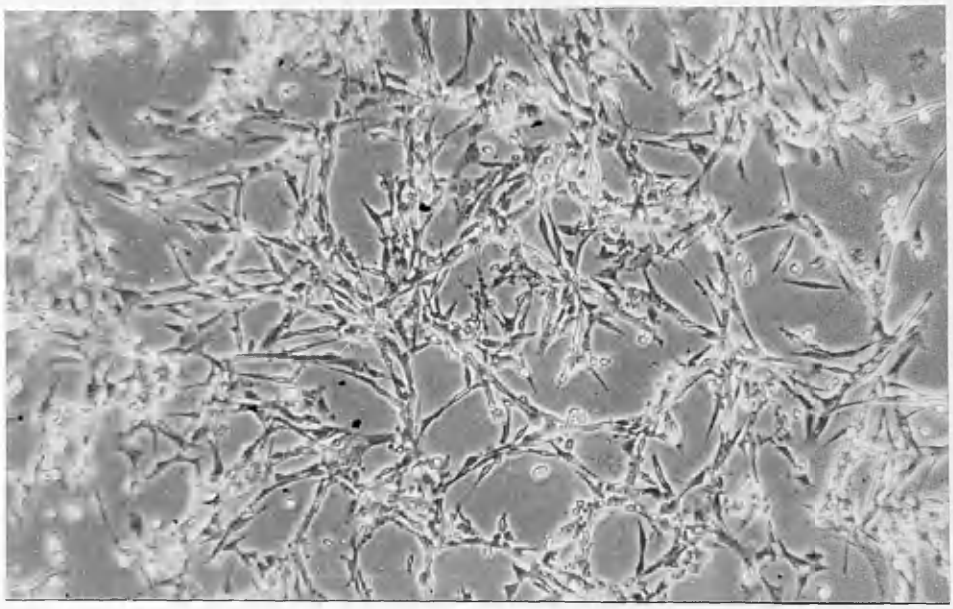
**CHAPTER 3:-** Analysis of changes in mannose-containing glycoproteins during the acquisition of fusion competence.

Introduction.

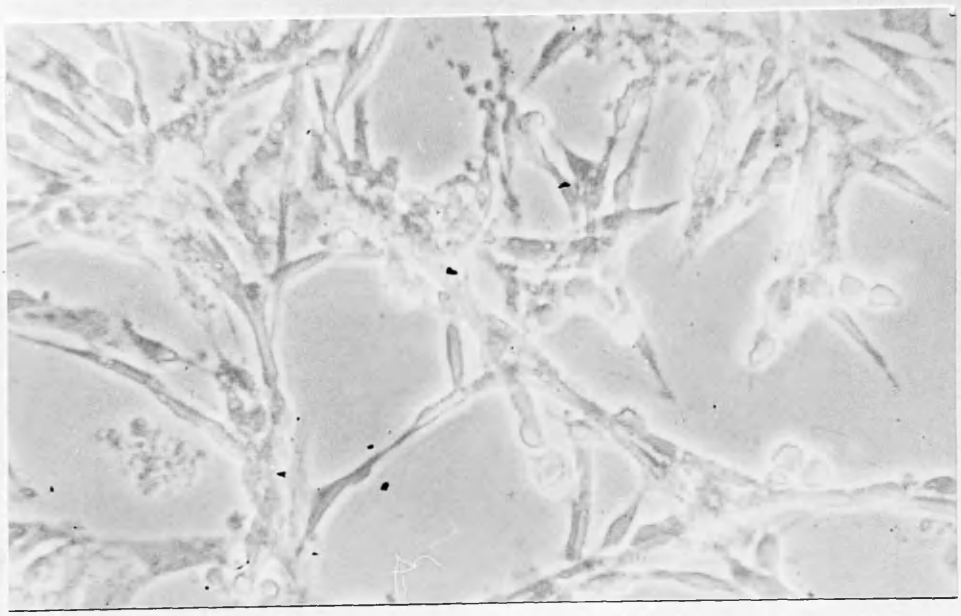
Myoblast fusion in vitro is practically over after 96 hours, although further differentiation of the fused myotubes will take place (Wakelam, 1985). By this time most of the cells have fused (Figures 1(g) and 1(h). Fusion, however, does not normally begin until the cells have been in culture for 48 hours or so. Figures 1(a) to 1(h) show this transition from mononucleate myoblast to multinucleate myotube. Under the microscope 24 hour old myoblasts (Figures 1(a) and 1(b)) and 48 hour old myoblasts (Figures 1(c) and 1(d)) are difficult to distinguish from each other. Yet while the 48 hour old myoblasts go on to form myotubes during the next 24 hours, the 24 hour old ones do not. The question then arises as to what changes take place within this time period that allow the 48 hour old myoblasts to fuse?

Previous investigators have examined the differences between pre- and post fusion myoblasts (Burridge and Jordan, 1980; Walsh and Philips, 1981) looking for specific proteins that may allow recognition and ultimately fusion. Surely, however, the change conferring fusion competence must take place before the cells actually fuse. Also, in these studies cell lines were used. As cell lines are known to behave differently from primary cells, a certain amount of caution has to be observed when interpreting the results. Further, if myoblast fusion is regulated by a protein, it is likely to be a mannose-containing glycoprotein, as Con A has been shown to inhibit fusion (Den et al., 1975; Sandra et al., 1977). As mentioned earlier, however, most glycoproteins are likely to contain

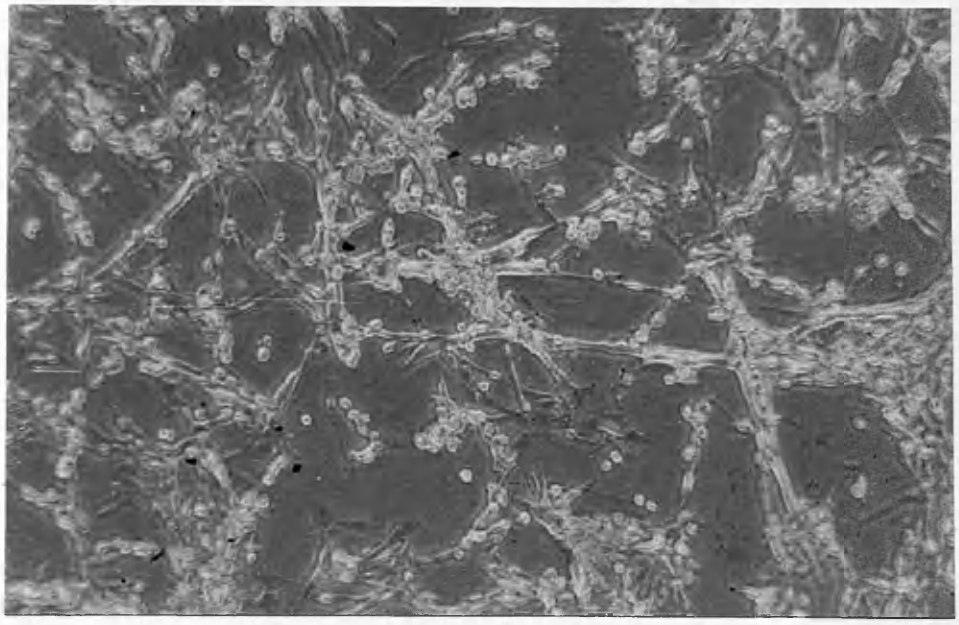
Figure1:- The fusion of myoblasts in vitro.



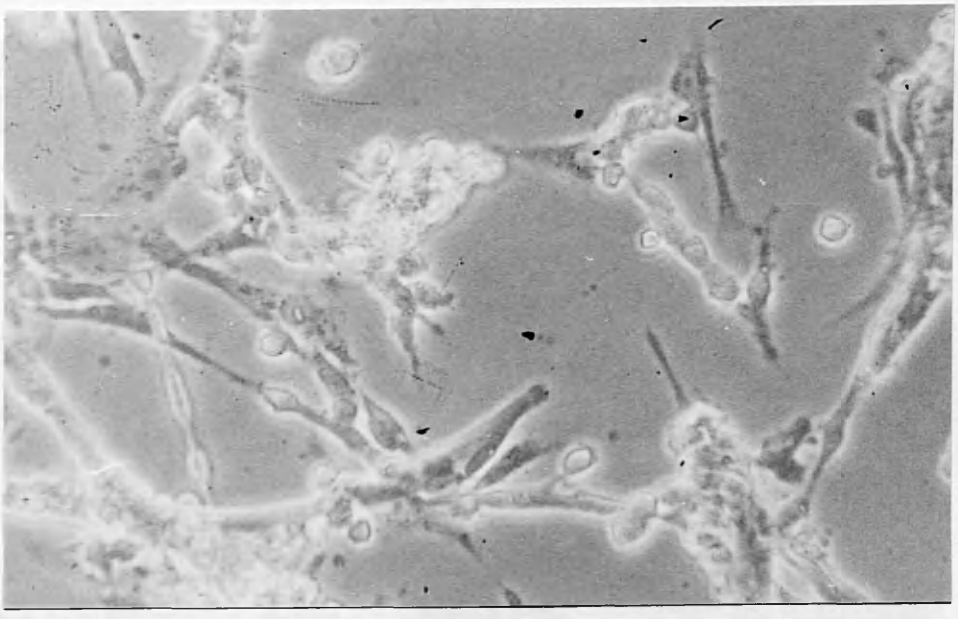
(a) Myoblasts after 24 hours in culture. Cells are undergoing division, and alignment of the cells has started.
(50x magnification).



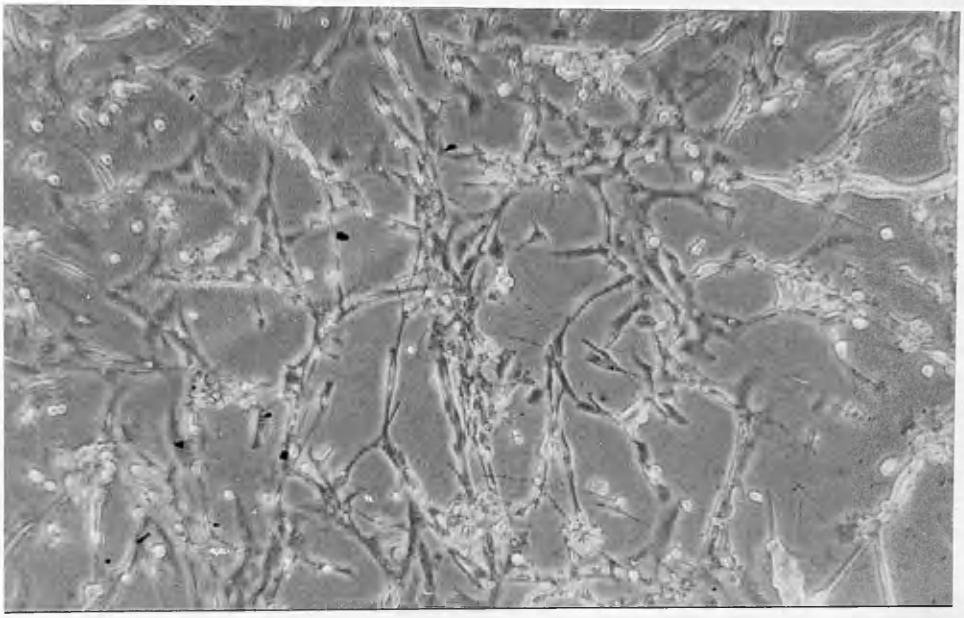
(b) As (a). (160x magnification).



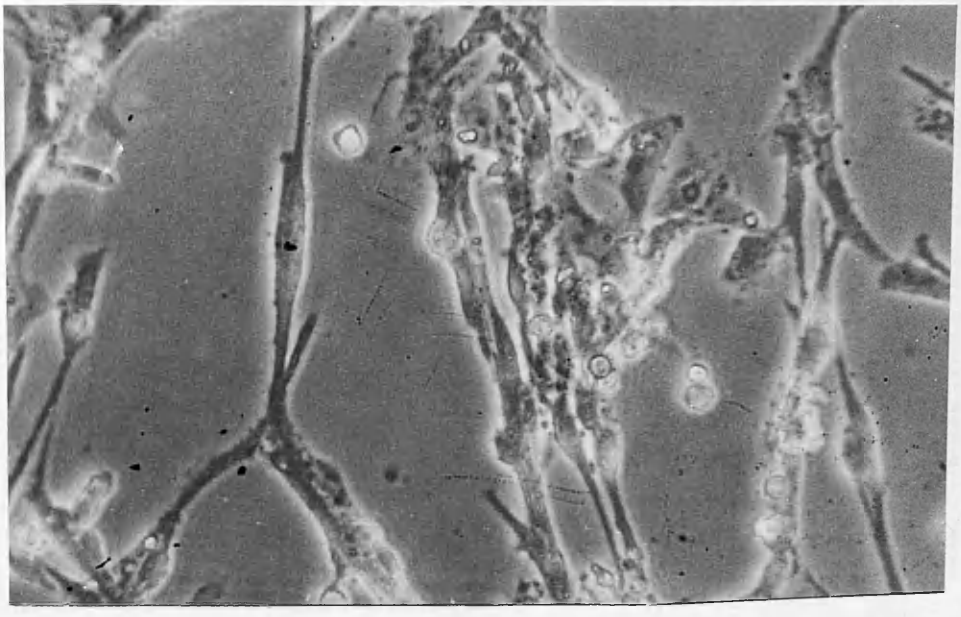
(c) After 48 hours in culture:- Extensive alignment has taken place, as has the beginning of fusion.
(50x magnification).



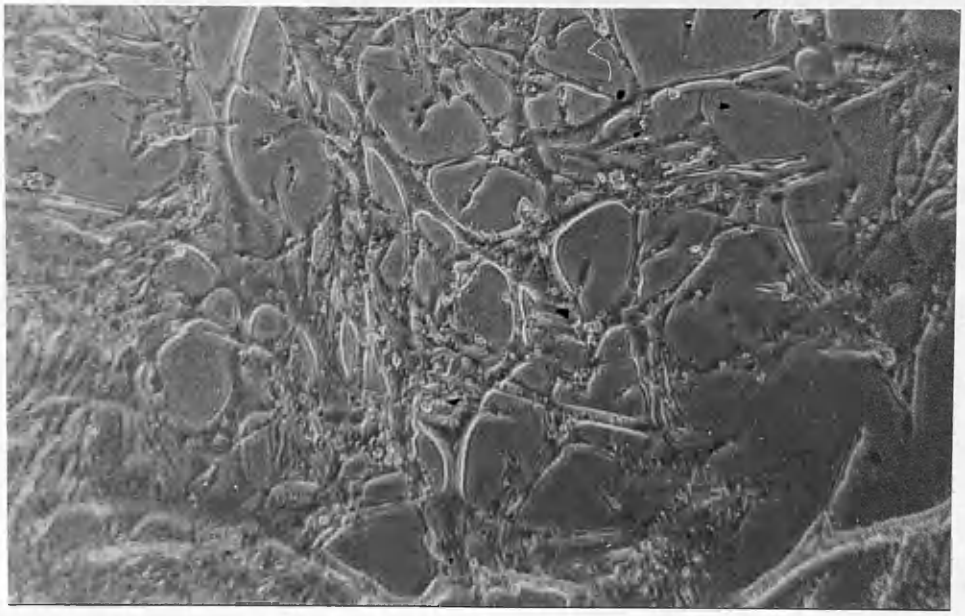
(d) As (c). (160x magnification).



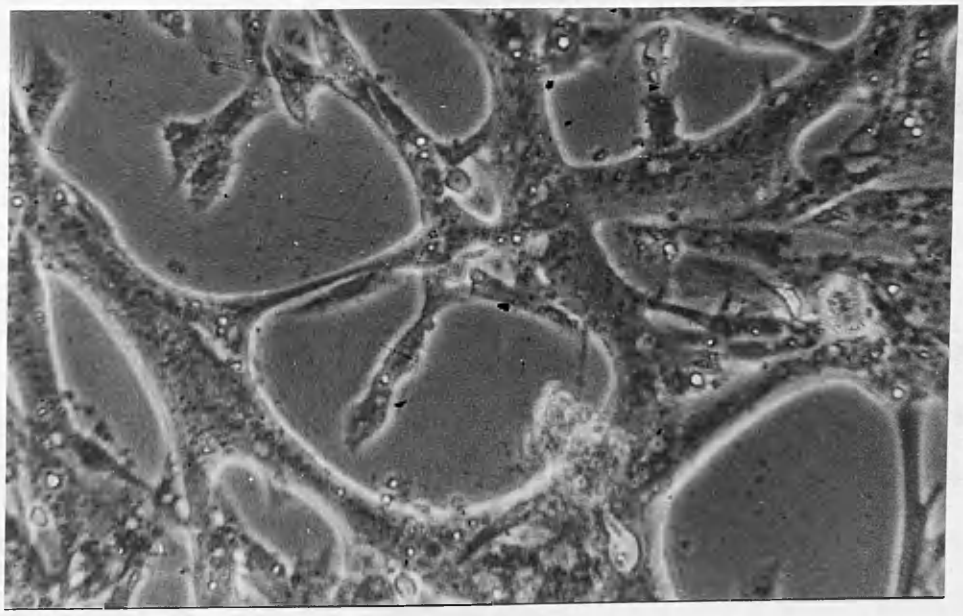
(e) After 72 hours in culture:- Fusion is well underway, with a number of small myotubes being readily apparent.
(50x magnification).



(f) As (e). (160x magnification).



(g) After 96 hours in culture:- Fusion is complete. As can be seen, the cells have increased in size quite dramatically. (50x magnification).



(h) As (g). (160x magnification).

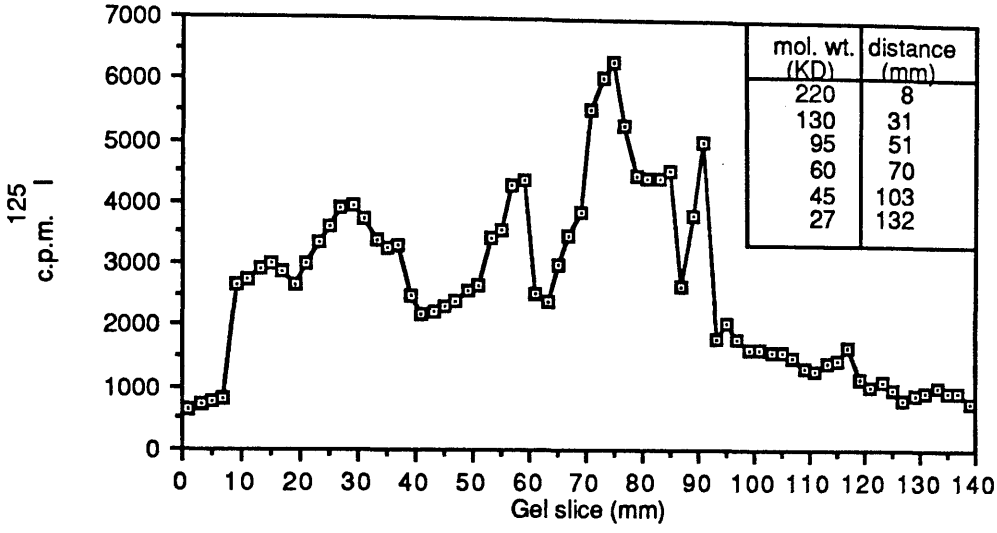
mannose (Hughes, 1983). Accordingly, the differences in mannose-containing glycoproteins between 24 hour old and 48 hour old myoblasts have been investigated. Iodinated Con A, in conjunction with SDS-PAGE and IEF, have been used in both one and two-dimensional analyses of the mannose-containing glycoproteins. Although this method does not exclusively label cell surface proteins, it is likely to label most of them as they are usually N-glycosylated (Bretscher and Raff, 1975; Kornfeld and Kornfeld, 1980; Hughes, 1983).

Use of SDS-PAGE.

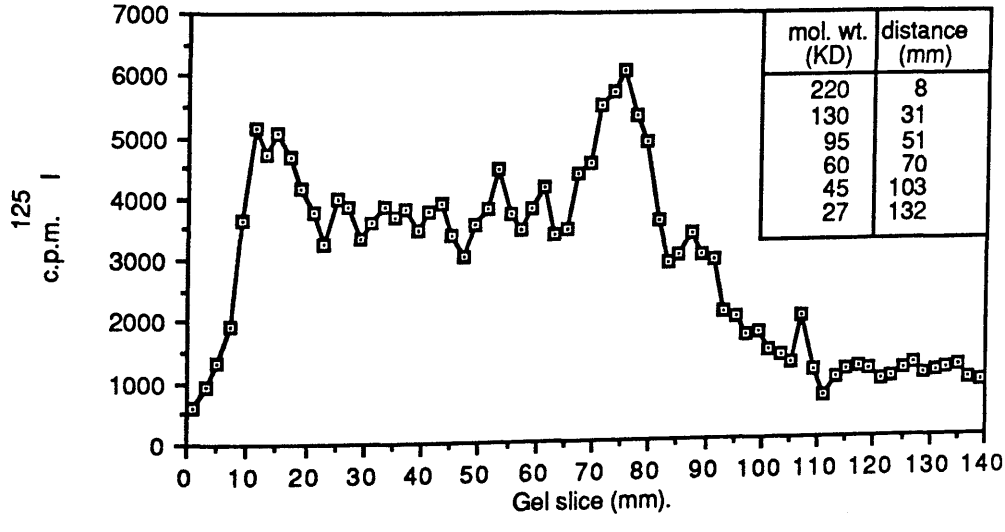
Identical amounts of whole cell material prepared for SDS-PAGE from 24 and 48 hour old (in vitro) chick skeletal myoblasts were separated on a 10% polyacrylamide gel. The gel was then immersed in a bath of iodinated Con A. After washing, each track was then cut into 1mm slices. The slices were then placed in a gamma counter and measured for $^{125}I$ activity. Two consecutive slices gave rise to each point on the graph. Figures 2(a) and (b) show the profiles of the mannose-containing glycoproteins of 24 hour and 48 hour old myoblasts respectively.

The major Con A binding protein in each case is one with an apparent molecular weight of 58KD. This protein, or group of proteins, appears to remain unchanged in its ability to bind Con A during the change from dividing myoblasts to the emergence of fusion competent ones. In order to aid comparison between the profiles, Figure 3 indicates the % change in the binding of $^{125}I$-Con A to each pair of gel slices. The % change was calculated using the following formula:-

Figure 2:- Iodinated Con A binding profiles to myoblast proteins separated by SDS-PAGE.




(a) The figure shows the binding profile of $^{125}$I-Con A to proteins from 24hr old chick skeletal myoblasts which were separated on a 10% polyacrylamide gel. The table in the top right-hand corner indicates the distance travelled by the molecular weight markers.




(b) As (a), except proteins from 48hr old chick myoblasts were used.

Figure 3:- Comparison of the $^{125}$I-Con A binding profiles shown in Figure 2.

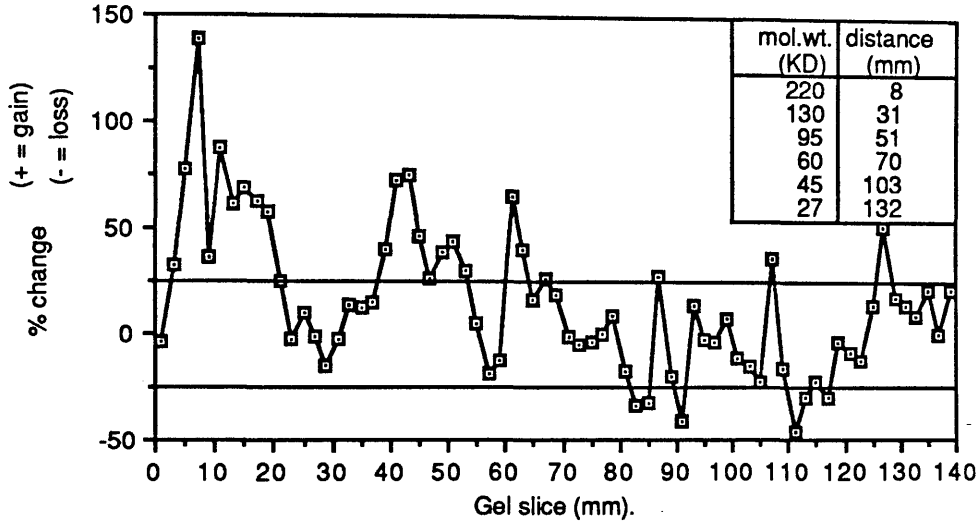



Figure 3 indicates the % change in the binding of Con A to each pair of gel slices. The % change was calculated using the following formula:-

$$\frac{\text{(cpm from 48hr gel slice) - (cpm from 24hr gel slice)}}{\text{(cpm from 24hr gel slice)}} \times 100$$

A positive number indicates an increase in the amount of $^{125}$I-Con A binding, and a negative number indicates a decrease. The table in the top-right hand corner indicates the position of the molecular weight standards.

$$\frac{\text{(cpm from 48 hour gel slice) - (cpm from 24 hour gel slice)}}{\text{(cpm from 24hr gel slice)}} \times 100$$

Therefore a positive number indicates an increase in the amount of the Con A binding protein, and a negative number indicates a decrease.

Approximately 60% of the points on the graph lie within $\pm$ 25% of the origin, indicating that there is little change in the synthesis of the majority of the Con A binding proteins. The most notable change occurs at the molecular weight 220KD, a molecular weight that corresponds to that of fibronectin. Here an increase of 140% takes place. Other notable increases include glycoproteins of molecular weight 190KD (88%), 180KD (69%), 120KD (75%), 100KD (43%), 88KD (64%), 40KD (36%) and 28KD (51%). The figures in parentheses indicate the % change. There are relatively few glycoproteins that decrease in amount, and those that do are in the lower molecular range. Those that decrease are of molecular weight 54KD (-34%), 50KD (-41%), and 37KD (-45%).

What these glycoproteins actually do in terms of function is a matter for conjecture. One would expect that the loss of a protein would be associated with a loss of a particular function. Similarly, an increase in a particular protein would be associated with a rise in a particular function. For example, as the 48 hour old myoblasts are post-mitotic, the loss of the 54KD, 50KD, and 37KD proteins could be associated with the loss of the capacity to divide. Similarly, the increase in the

proteins could be associated with the function of initiating fusion.

Due to the insensitivity of this assay the appearance of new proteins and the disappearance of old ones are difficult to detect. The separation of the proteins by SDS-PAGE is rather limited when dealing with the large number of proteins present in whole cells, resulting in the overlapping of proteins. Also, this method does not separate different proteins of the same molecular weight.

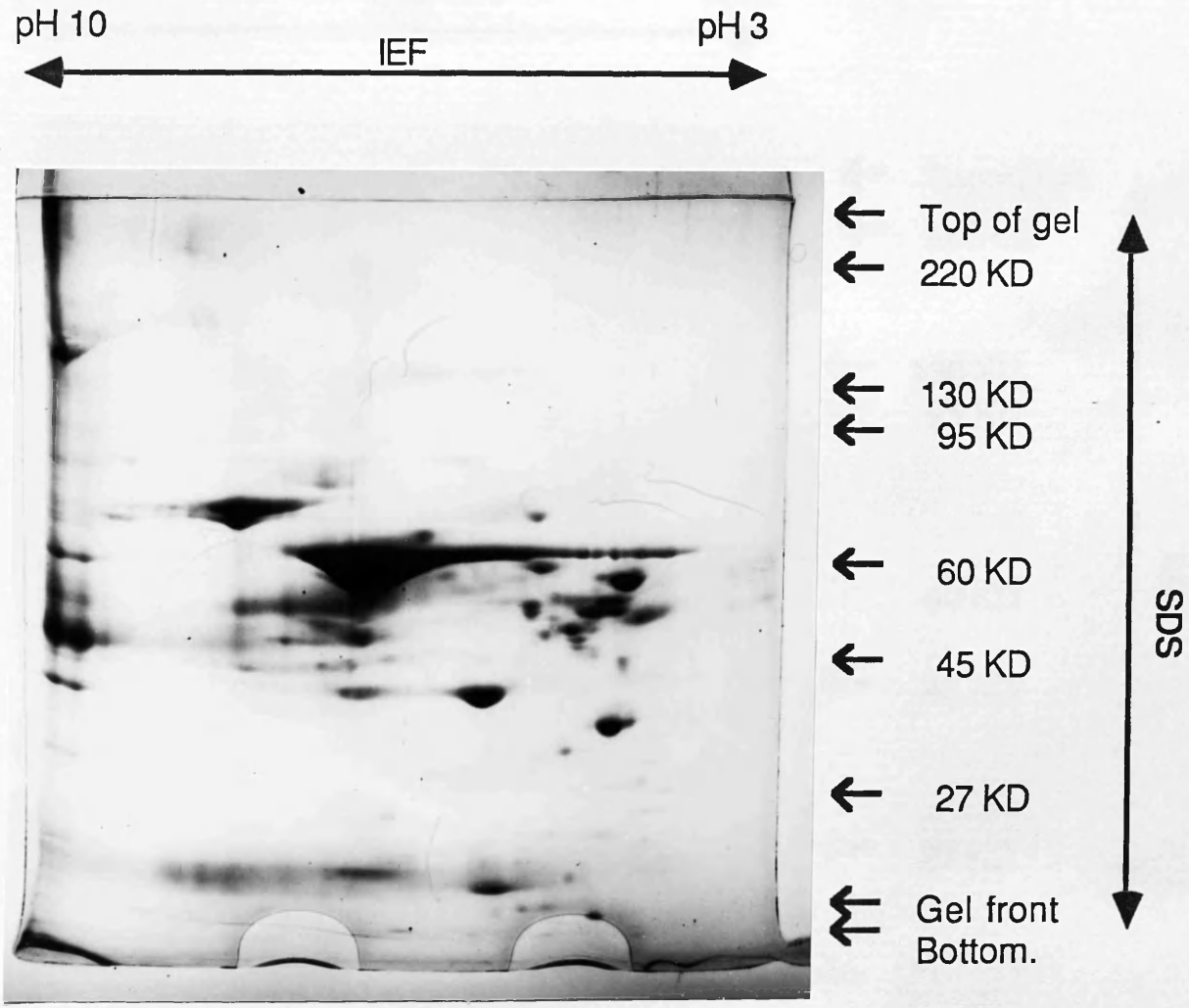
Use of 2D-PAGE.

A much better separation can be acheived using 2D-PAGE, where the proteins are first separated by charge, and then by molecular weight. Figures 4(a) to 4(f) show the patterns obtained using 24 hour and 48 hour old myoblasts. While these patterns from 2D-gels give more information than the 1-D gels, they can be more difficult to interpret.

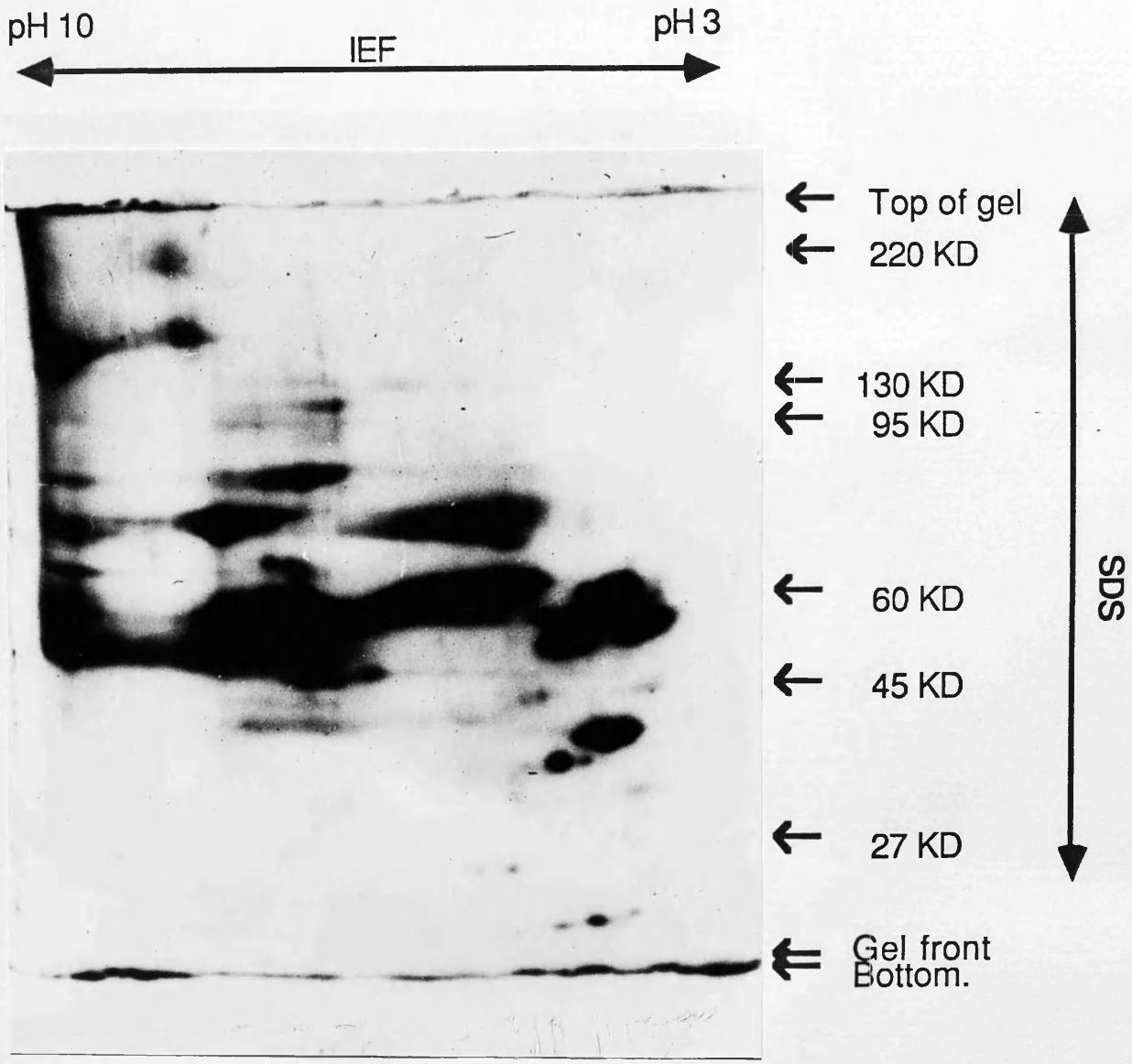
Perhaps the most notable feature of the gels and their respective autoradiographs is that the major Coomassie Blue binding protein fails to bind any $^{125}$I-Con A. This protein has an apparent molecular weight of approximately 60-65KD, which corresponds to that of serum albumin. It is probable, as the myoblasts are grown in a serum-containing medium, that this protein represents the amount of albumin binding to the cell surface. The size and shape of the spot indicates that it is present in large quantities (see O'Farrell, 1975). Also serum albumin is known not to contain mannose.

As with the profiles shown in Figure 2 the autoradiographs of the 2D-gels indicate the major Con A

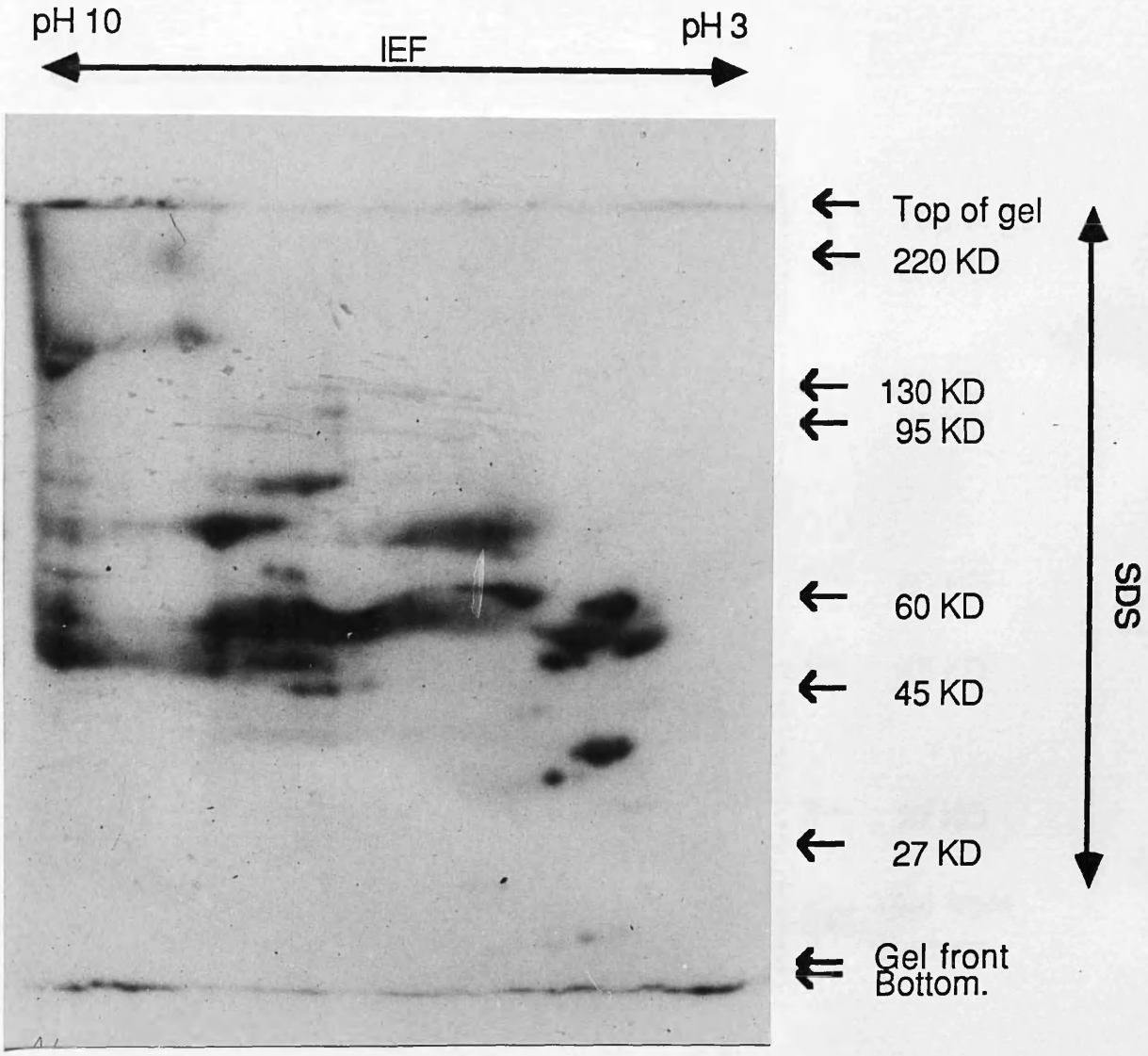
Figure 4:- Two-dimensional analysis of iodinated Con A binding proteins.



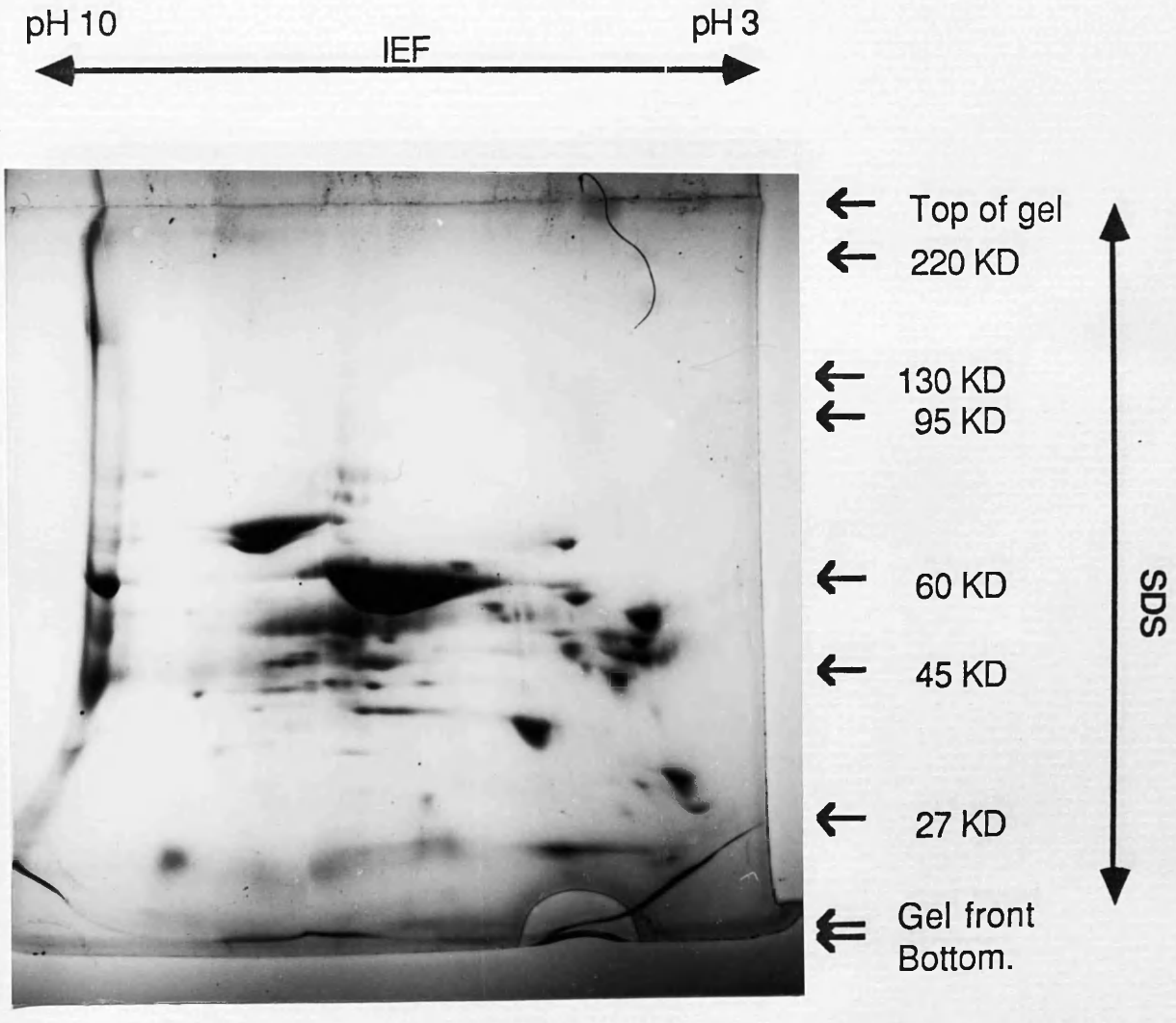

(a) 2D-gel of 24 hour old myoblasts. Coomassie blue stain.



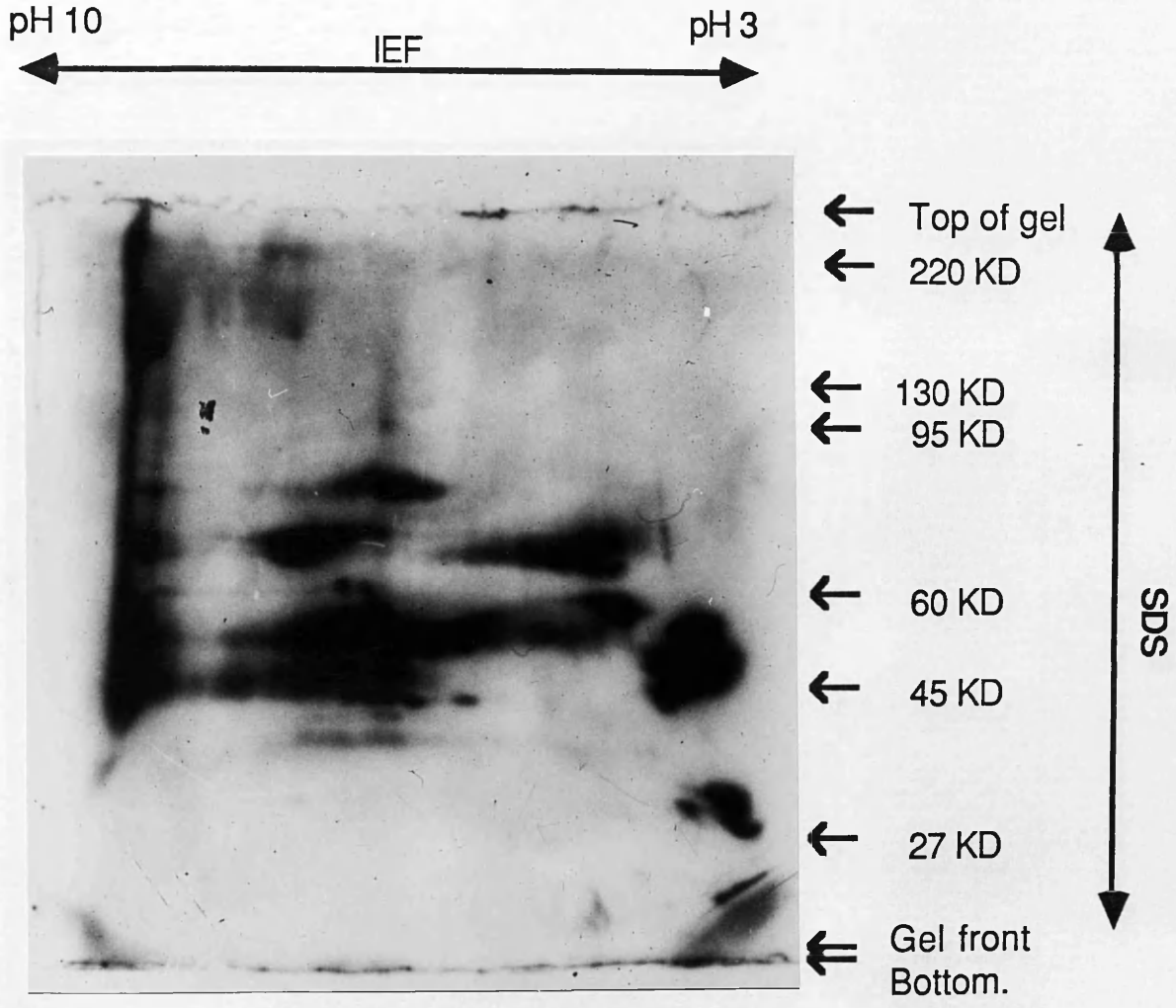

(b) Autoradiograph of (a). Exposure time = 24 hours.



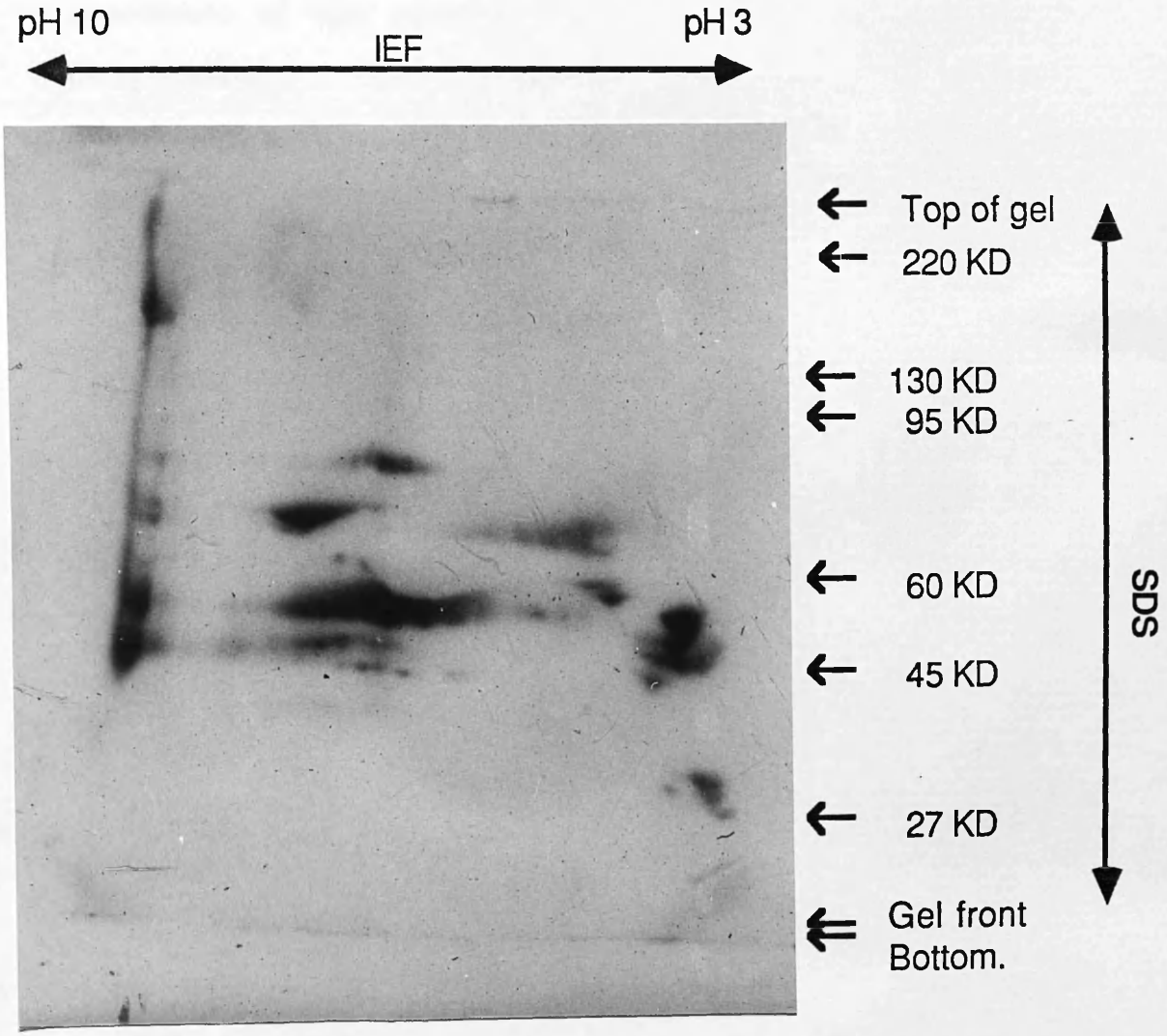

(c) Autoradiograph of (a). Exposure time = 6 hours.




(d) 2D-gel of 48 hour old myoblasts. Coomassie blue stain.




(e) Autoradiograph of (d). Exposure time = 24 hours.



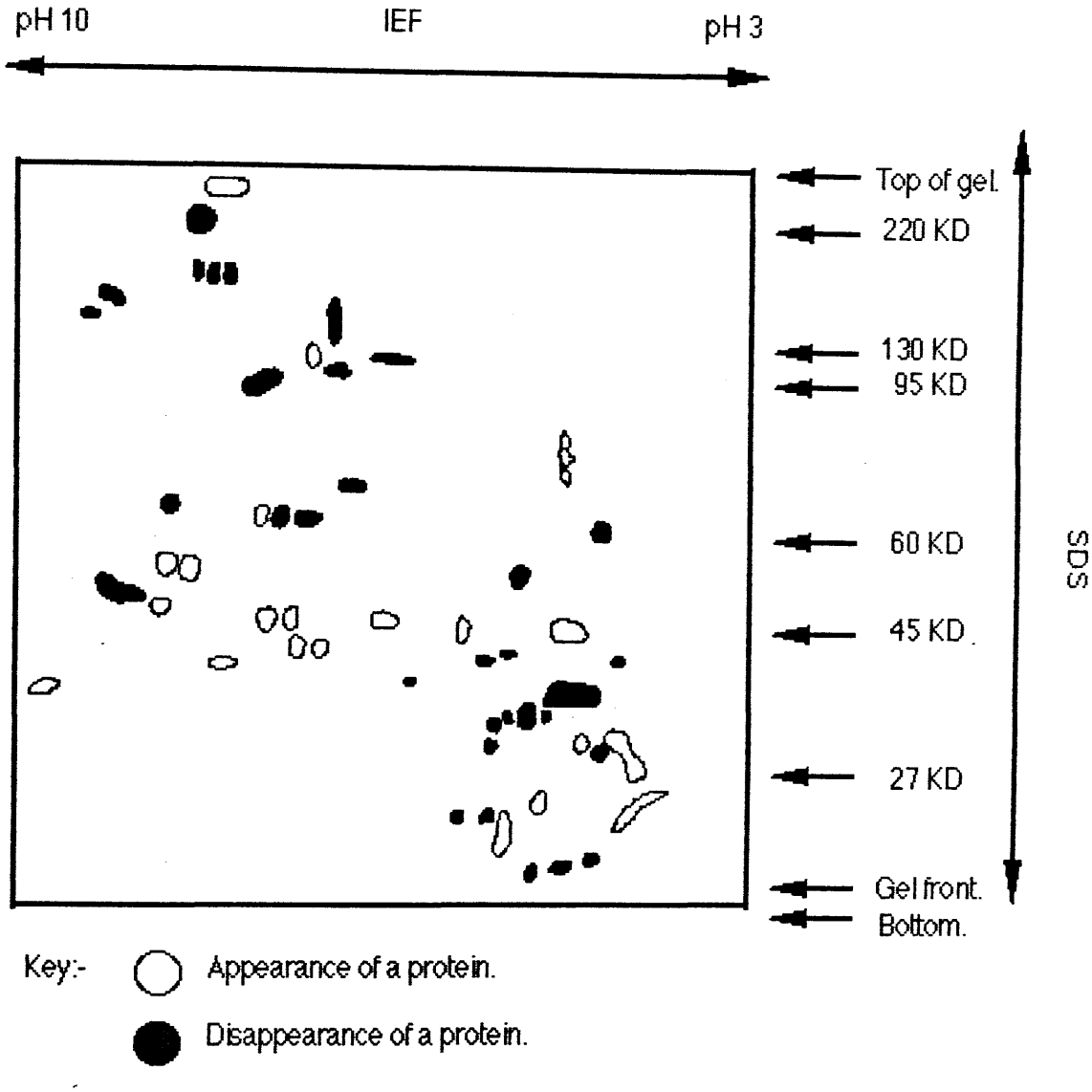

(f) Autoradiograph of (d). Exposure time = 6 hours.

binding proteins to be between 45KD and 60KD, and that Con A binds mainly to the higher molecular proteins (>40KD). Figure 5 indicates the appearance and disappearance of mannose-containing glycoproteins. The open spots indicate the appearance of new proteins, while the filled spots indicate a disappearance. The comparison only takes into account qualitative, and not quantitative changes. The diagram is meant only to serve as an indication of the relative position of the spots and not the relative amounts.

As can be seen the pattern of change is much more complicated than is suggested by the 1D-separation (Figure 3). At least 33 different glycoproteins have been lost, coupled with the appearance of at least 23 new glycoproteins. Then there are the quantitative changes to take into consideration on top of that. Strictly speaking Figures 3 and 5 are not comparable due to the nature of the assays. Figure 3 is designed to show quantitative changes while Figure 5 shows qualitative changes. While Figure 3 indicated a rise in synthesis of proteins of molecular weight 190KD, 180KD, 120KD, and 100KD, Figure 5 shows mainly a loss of proteins in that molecular weight range. This serves to indicate how different approaches can give rise to different results.

It would be impossible to attribute a function to each protein. What it does indicate, however, is that there are major changes taking place when the myoblast moves from being fusion incompetent to being fusion competent. Intuitively, at least one of these changes (and probably several) must be involved with the new function of myoblast recognition and fusion. The question would then be, which one(s)?

Figure 5:- Comparison of the autoradiograph shown in Figure 4.




This diagram displays the relative positions of 125I-Con A binding proteins which appear and disappear during the emergence of fusion competent cells. The comparison only takes into account qualitative and not quantitative changes.

The next two chapters are involved with trying to narrow down the choice.

**CHAPTER 4:-** Affinity chromatography of myoblast membrane components.

Introduction.

Affinity-chromatography usually involves the binding of a protein which is capable of eliciting a specific interaction, such as a lectin or immunoglobulin, to supporting structure such as Sepharose, which is then packed into a column. When a solution containing a mixture of compounds is passed over the column, only compounds possessing the correct features will bind to the column. For example, those containing mannose when Con A is used, or the antigen against which the antibody was raised when using IgG, The substances binding to the column can then be eluted in various ways. Here, a novel approach to affinity-chromatography was undertaken. If cell-cell adhesion is mediated by interactions between specific proteins at the cell surface, then perhaps these proteins could be isolated by a form of affinity chromatography (which is outlined below and described in detail in Chapter 2).

Plasma-membranes were isolated from 48 hour old (in culture) myoblasts (deemed to be fusion competent), the proteins extracted in the presence of octyl-glucoside and bound to CN-Br-activated Sepharose. Further 48 hour old myoblasts were cell surface iodinated with $^{125}I$, solubilised in the presence of octyl-glucoside, and passed over the column. The column was then washed extensively with running buffer and the bound proteins were eluted with a salt gradient (see Materials and Methods for full details). In this way it should be possible to isolate cell-surface proteins that are capable of adhering to each other.

Results.

Iodinated cell-surface proteins were added to the column under two different conditions:-

(a) In the absence of calcium, and

(b) In the presence of 1.6mM calcium (this concentration has been found to be the optimum for myoblast aggregation (Knudsen and Horowitz, 1979)).

In the absence of calcium, the iodinated material added to the column failed to bind (or at least no iodinated material came off the column after the addition of 1M $Na^+Cl^-$, nor when the column was washed with 3M $Na^+Cl^-$. Also, the use of a hand-held gamma counter failed to detect any increase in radioactivity). In the presence of $Ca^{2+}$ (1.6mM), however, a large amount of iodinated material was eluted from the column, with the bulk of the protein being eluted between 0.16 and 0.4M $Na^+Cl^-$. The elution profile is shown in Figure 6. As can be seen from this figure there are two main peaks, one showing slightly lower affinity binding than the other.

The amount of iodinated material eluted represents a very small fraction of the material added to the column (<0.1%). Fractions 8-24 were force dialysed to a small volume (200ul) and prepared for SDS-PAGE. The proteins were then separated on a 10% polyacrylamide gel. No bands were detected by the use of Coomassie Blue stain, indicating that the amount of protein present was rather small. However, when the gel was dried and exposed to X-ray film, the autoradiograph detected the presence of nine distinct bands, of molecular weights 250KD, 225KD, 170KD, 140KD, 100KD, 76KD, 58KD, 41KD, and

Figure 6:- Elution profile of bound proteins.

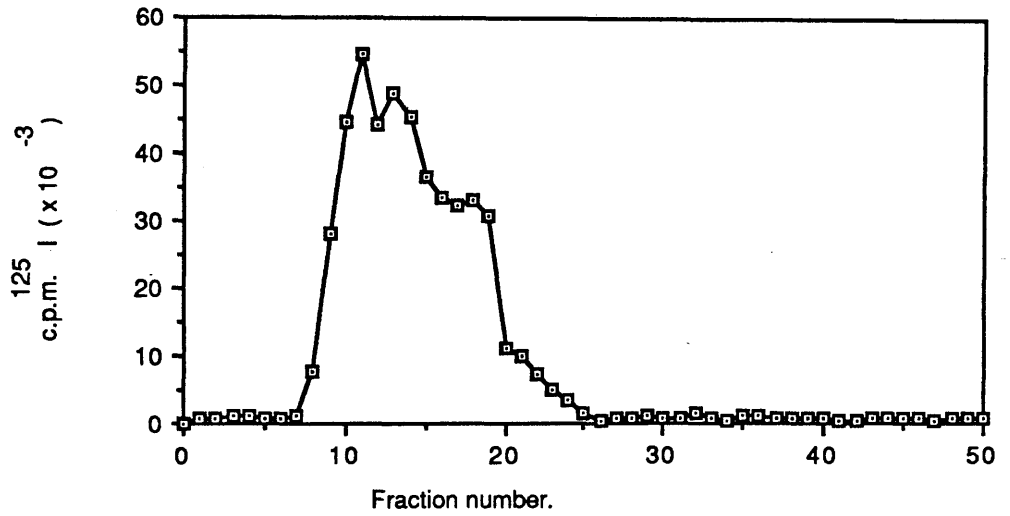



Figure 6:- The bound proteins were eluted using a gradient of 0-1M $Na^+Cl^-$. Two ml fractions were collected and 100ul samples were taken from each and tested for $^{125}I$-activity. The two main peaks were eluted in concentrations of 0.24M and 0.28M $Na^+Cl^-$ respectively.

29KD (see Figure 7; a densitometric scan of the autoradiograph is also shown).

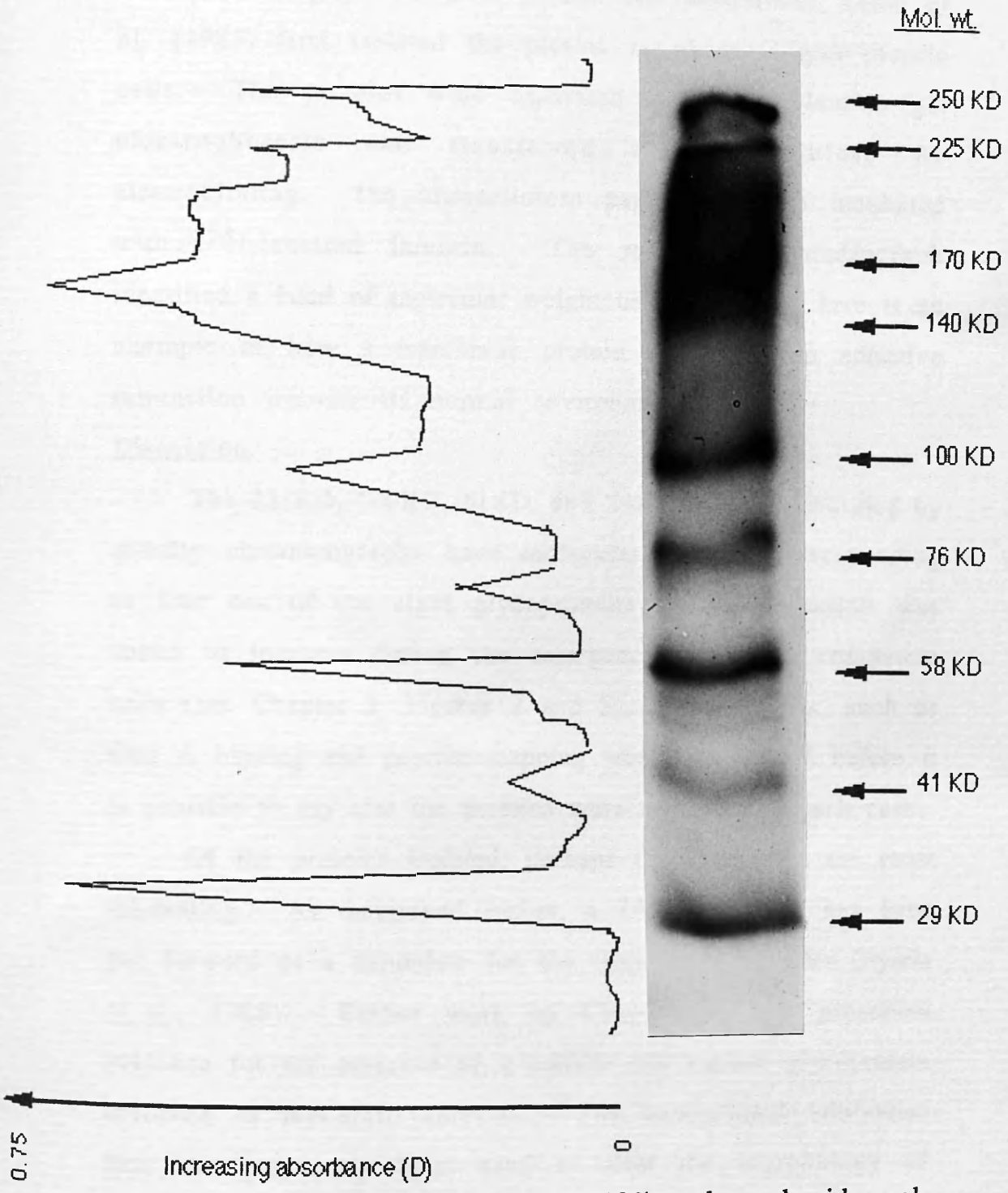
Justification of experiment.

Before discussing the likely candidates the proteins represent, it is perhaps pertinent to discuss first of all the validity of the experiment. After all, the cell surface proteins have been removed from their natural environment and have been asked to perform their usual function, that is, binding.

There are some precedents in the literature to justify this type of experiment. Firstly, affinity chromatography was used to identify a putative cell surface receptor for fibronectin by Pytela et al. (1985). Using the 120KD chymotryptic cell binding of fibronectin as the affinity matrix, they passed octylglucoside extracts of MG-63 human osteosarcoma cells over the column. Specific elution was effected by using a synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro, which contains the "cell binding" tripeptide of Arg-Gly-Asp. In this way a 140KD protein was isolated. Further tests revealed this 140KD protein to be a glycoprotein, and suggested that it was a "membrane-embedded cell surface protein directly involved in the initial step of cell adhesion to fibronectin substrates".

Obviously this is a much more controlled experiment than the one described in this chapter, due mainly to the known biochemistry of fibronectin. What is important to note though, is the 140KD "membrane-embedded cell surface protein", despite being removed from its natural environment, was still able to adhere to the 120KD chymotryptic cell binding fragment of fibronectin.

Figure 7:- Autoradiograph of proteins isolated by affinity chromatography, plus accompanying densitometry scan.




The proteins were separated on a 10% polyacrylamide gel which was then dried and exposed to X-ray film. The resultant autoradiograph was scanned using a Joyce-Loebl densitometer.

There are numerous examples of membrane proteins still being able to elicit some function when removed from the plasma membrane. For example, in an attempt to isolate a laminin binding protein from muscle cell membranes, Lesot et al. (1983) first isolated the plasma membranes from muscle cells. The proteins were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. The nitrocellulose paper was then incubated with $^{125}$I-labelled laminin. The resultant autoradiograph identified a band of molecular weight 68KD. Again, here is an example of how a membrane protein can elicit an adhesive interaction outwith its normal environment.

Discussion.

The 225KD, 100KD, 41KD, and 29KD proteins identified by affinity chromatography have molecular weights corresponding to four out of the eight glycoproteins whose expression was found to increase during the emergence of fusion competent cells (see Chapter 3, Figures 2 and 3). Further tests, such as Con A binding and peptide mapping would be needed before it is possible to say that the proteins were the same in each case.

Of the proteins isolated, perhaps the 140KD is the most interesting. As mentioned earlier, a 140KD protein has been put forward as a candidate for the fibronectin receptor (Pytela et al., 1985). Earlier work by Chapman (1984) presented evidence for the presence of a 140KD cell surface glycoprotein involved in myoblast adhesion. Two monoclonal antibodies that had previously been used to alter the morphology of cultured chick myogenic cells (Wylie et al., 1979) were both found to interact with the same 140KD protein. During

immunoprecipitation of this protein by the antibodies, a second protein of 170KD was also found in the precipitate. Chapman (1984) concluded that these proteins were probably unrelated as their peptide maps were quite disimilar. Earlier still, Knudsen et al. (1981) isolated a group of proteins with molecular weight 140KD involved in the adhesion of hamster cells to the substratum.

Chen et al. (1986) have suggested that an 140KD protein acts as a linkage between fibronectin and the cytoskeleton in chick lung cells. Using double and triple labelling experiments, they found that the 140KD protein complex interacts and colocalizes with both fibronectin and microfilament bundles. It was further proposed that the 140KD protein complex interacts with fibronectin to provide the " labile, motility-related cell adhesion necessary for morphogenetic movements". N-CAM also has a molecular weight of 140KD in SDS (Thiery et al., 1977). Also as mentioned earlier, chicken N-CAM was found to be composed of proteins of molecular weight 170KD and 140KD that arise by alternate splicing of mRNA from a single N-CAM gene (Murray et al., 1986), although these molecular weights represent the adult form of the molecule. The embryonic form appears as a diffuse band at around 220KD in SDS-PAGE.

It is quite possible that the140KD band represents a group of proteins of the same molecular weight. The fact that bands of 250KD and 225KD were also isolated and that these bands correspond to the molecular weight of fibronectin lends evidence to the possibility that the 140KD protein may be the fibronectin receptor. It is possible that fibronectin could come from two sources. Firstly, the myoblasts are known to produce

their own, albeit at a reduced amount (Chiquet et al., 1981), or alternatively the cells could bind fibronectin present in the serum. In either case it seems likely that some fibronectin would end up in the plasma membrane fraction during isolation.

Three of the other proteins isolated by affinity chromatography could correspond to proteins found by other workers. Walsh and Philips (1981), using $^{125}$I-WGA binding to proteins separated by SDS-PAGE found a 100KD protein that was myoblast specific. Also by using $^{125}$I-Con A they found 76KD and 27KD proteins present on myoblasts and on myotubes at an early stage of differentiation.

One of the unfortunate problems associated with this affinity chromatography experiment is the amount of material isolated. From approximately $10^9$ cells (allowing for only three doublings in the 48 hours after plating out) which were cell surface labelled with $^{125}$I the amount of protein isolated was minuscule (<1ug), as Coomassie Blue failed to stain the gel. Therefore, in order to do any meaningful biochemical analysis, a vast number of cells would be required. Due to this limitation the proteins isolated were not investigated further by this method.

## CHAPTER 5:- Comparison of the adhesion of fusion incompetent and fusion competent myoblasts, and their inhibition of aggregation by monovalent Fabs.

Introduction.

In Chapter 3 it was established that there are a great number of changes taking place in the set of glycoproteins sythesised by myoblasts during the acquisition of fusion competence. Here it is reported whether or not these changes resulted in any differences in the adhesion and aggregation of fusion incompetent and fusion competent cells. Two different methods were employed.

Firstly, adhesion was measured by lawn collection assay (see Chapter 2), whereby 24 hour old myoblasts (fusion incompetent) and 48 hour old myoblasts (fusion competent) were seeded onto myoblasts of varying age. Adhesion is expressed as the number of cells attached (as a percentage of the total added) after 50 minutes. Secondly, antibodies were raised that were capable of recognising 48 hour old myoblasts. It was also hoped to use this antibody to distinguish between the adhesion phase and the recognition phase; that is, to produce an antibody capable of preventing fusion, but which allows adhesion to take place.

Results.

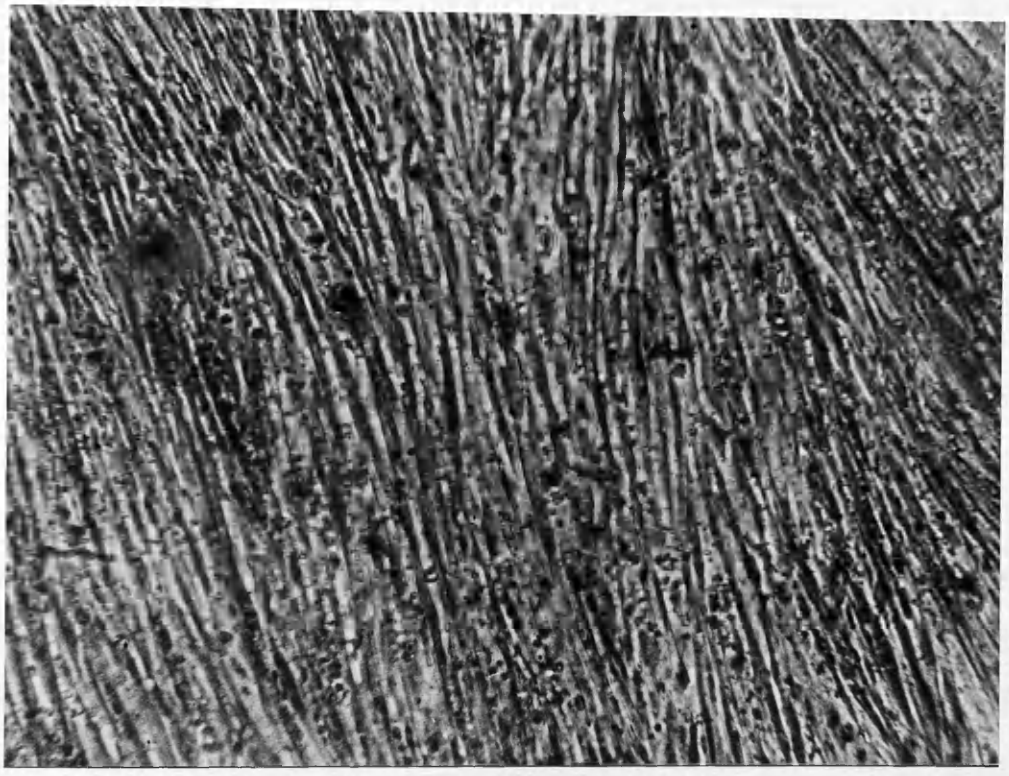
It was found that long term cultures of myotubes (2-3 weeks) had a tendency to "curl up" at the edges, and become detached from the tissue culture dish, when grown on gelatin-coated substrata. In order to prevent this, myoblasts and myotubes were grown on Type I collagen gels (Schor, 1980). Not only did the myotubes adhere better to this substratum over the longer time period, but they also displayed a remarkable orientation (Figure 8). Presumably this reflects the

orientation of the collagen fibres, which probably align during the gelling process.

Figure 9 compares the adhesion of the 24 hour old and 48 hour old myoblasts to short term cultures of myoblast/myotube monolayers (1-5 days old) in the presence and absence of serum, while Figure 10 compares their adhesion to the longer term cultures (1-3 weeks). As can be seen, there is practically no difference between the adhesion characteristics of the 24 hour old and the 48 hour old myoblasts. Whatever the culture condition, or whatever the age of the myoblast/myotube monolayers, the 24 hour old myoblasts display the same level of adhesion as the 48 hour old myoblasts. However, it appears that both the 24 hour and 48 hour old myoblasts adhere better to the longer term cultures than to the short term cultures, both in the absence and presence of serum ($P<2.5\%$ in each case, as measured by Student's t-test). Furthermore, it appears that the absence of serum doesn't affect the adhesion characteristics of the myoblasts to the myoblast/myotube monolayers (with the exception of the 3 week old monolayer, in which case both the 24 hour and 48 hour old myoblasts display an increased adhesion in the presence of serum ($P<5\%$, as measured by Student's t-test)).

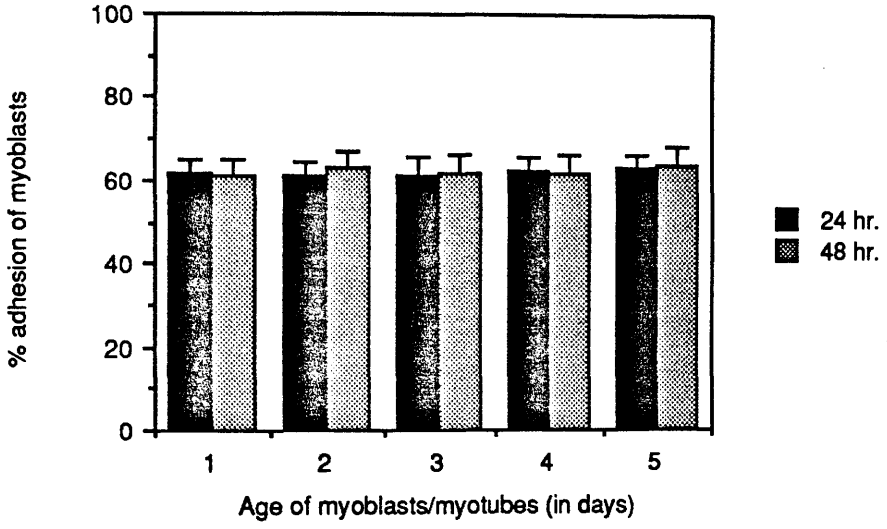
One criticism of this type of assay is that it does not measure the strength of adhesion, but merely whether or not the cell has become attached to the monolayer. It is quite possible that the 24 hour and 48 hour old myoblasts display different "strengths" of adhesion, and as such it would not be discovered by this assay. What it does show, however, is that

Figure 8:- Myotubes grown on top of a collagen gel.

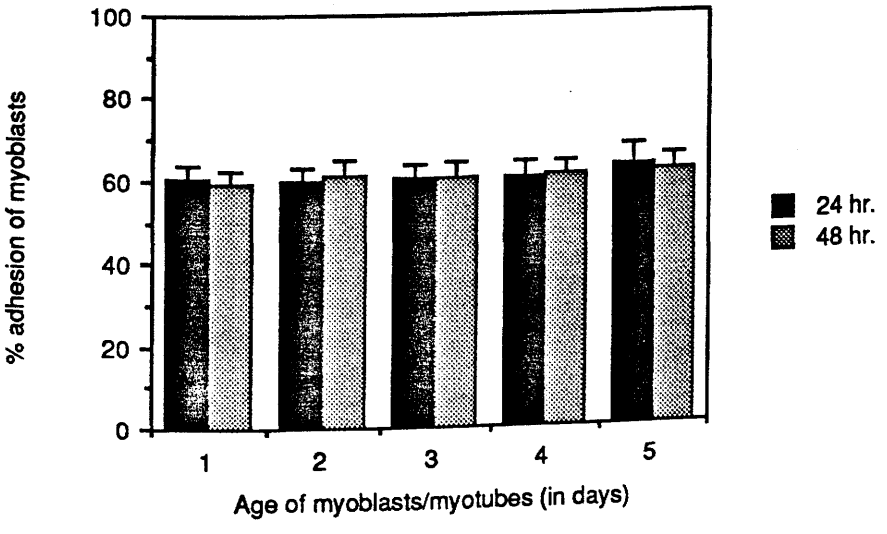


Ten day old myotubes were grown on the top of a gel made from type I collagen. The myotubes display a very noticeable orientation. In this case the myotubes are running from the top to the bottom of the photograph. (Magnification x40).

Figure 9:- Comparison of the adhesion of 24hr old and 48hr old myoblasts to short term cultures.

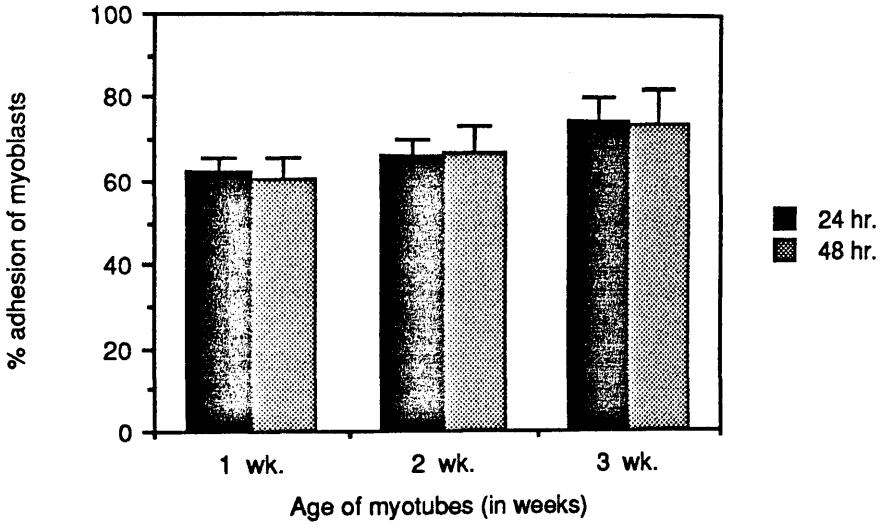



(a) 24hr old and 48hr old myoblasts were seeded onto myoblast/myotube monolayers of different ages in the presence of serum. Adhesion is expressed as the number of cells attached to the monolayer (as a % of the total added) after 50 minutes.

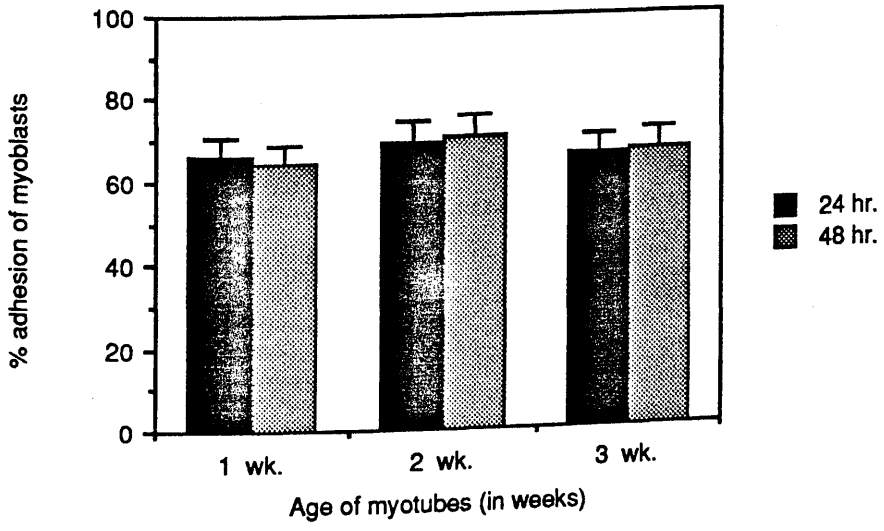



(b) As (a), but in the absence of serum.

Figure 10:- Comparison of the adhesion of 24hr old and 48hr old myoblasts to long term cultures.




(a) 24hr old and 48hr old myoblasts were seeded onto myotubes of different ages in the presence of serum. Adhesion is expressed as for Figure 9.




(b) As (a), but in the absence of serum.

both 24 hour old and 48 hour old myoblasts are capable of adhering to the myoblast/myotube monolayers to the same extent.

It is not particularly surprising that the extent of adhesion of the cells to the monolayer is independent of the presence or absence of serum (except for the adhesion of the cells to the three week old myotubes). After all, Puri and Turner (1978) and Chiquet et al. (1981) have shown that cell-cell adhesion and fusion can take place in the absence of serum, and therefore fibronectin. The observation that there is an increased adhesion when the myoblasts are seeded onto 3 week old myotubes may be explained by examining the surface coat of the myotubes. As the myotubes age, they develop a thick sheath of material that can be stained by ruthenium-red (which stains sugars) (Bischoff, 1978). It is possible that this sheath prevents direct cell-cell contact, and that adhesion is mediated through some component found in serum.

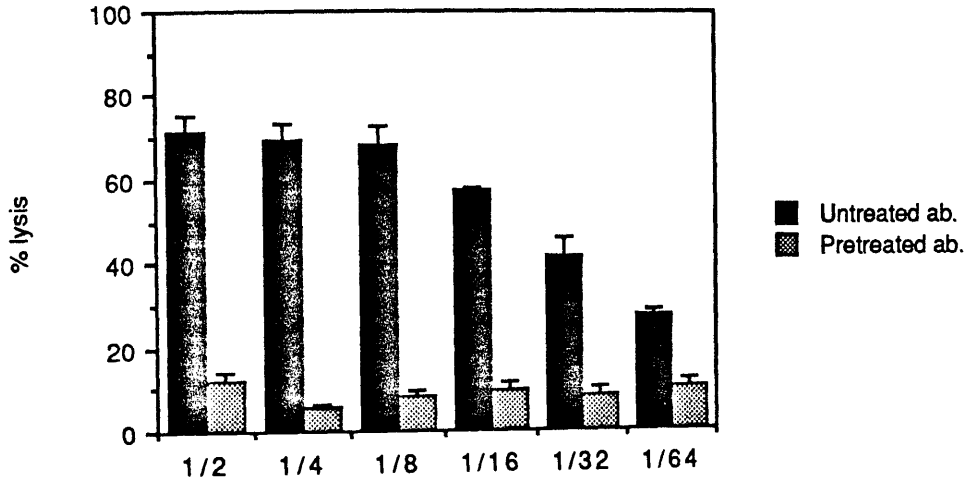
It was hoped that some change in adhesion would occur with the acquisition of fusion-competence. Unfortunately, the acquisition of fusion competence does not appear to result in any change in adhesion. It seems likely that there are multiple adhesive mechanisms (Nameroff and Munar, 1976), and that these mechanisms could mask the effects of a more specific adhesive mechanism (such as the mechanism involved in myoblast recognition). Therefore a more specific approach is required to identify such mechanisms. One such approach that has proved to be particularly useful in the identification of candidates for cell adhesion molecules has been the use of antibodies (see Chapter 1).

A rabbit was inoculated with 48 hour old myoblasts and IgG was isolated from the heat-inactivated immune sera by DEAE-cellulose chromatography. The antibody was split into two aliquots, and half was adsorbed extensively against 24 hour old myoblasts simply by adding the sera to monolayers of 24 hour old myoblasts (see Materials and Methods for full details). Initial adsorption caused the cells to round up, and these cells could easily be detached from the tissue culture flask by gentle shaking (presumably because the antibodies interfered with the antigens involved with cell-substrate adhesion). Eventually the adsorbed antibody lost the ability to do this. Both the pretreated antibody (P-ab) and the untreated antibody (U-ab) were tested for their reactivity against 24 hour and 48 hour old myoblasts by $^{51}Cr$-release cytotoxicity assay.

Figure 11 (a) shows that U-ab reacts strongly with 24 hour old myoblasts, causing lysis of over 70% of the cells in the presence of complement, while nearly all the reactivity against the 24 hour old myoblasts has been removed by the pretreatment of the antibody. On the other hand Figure 11 (b) shows that the pretreatment of the antibody does not affect its reactivity with the older myoblasts. This indicates that the 48 hour old myoblasts have at least one antigen unique to their cell surface, and in all likelihood have several, thereby confirming the results obtained in Chapter 3.

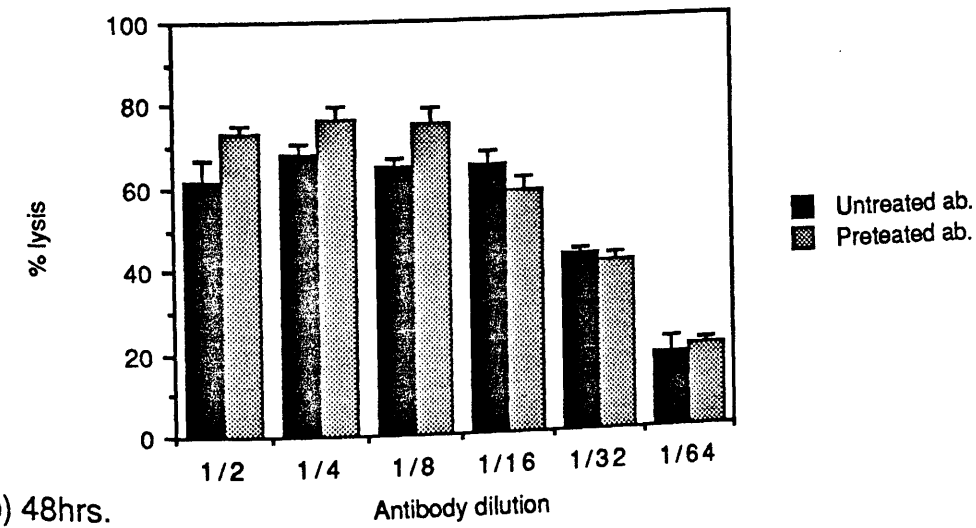
As mentioned previously, 48 hour old myoblasts are fusion competent, whereas 24 hour old ones are not. To test whether the appearance of the unique antigen(s) on the surface of 48 hour old cells were involved in fusion in any way, Fab

Figure 11:- Comparison of untreated and pretreated antibody directed against 24hr and 48hr old myoblasts.




(a) 24hrs.

The pretreated antibody is unable to cause lysis of the 24hr old myoblasts, indicating that most of the activity has been removed by adsorption.




(b) 48hrs.

The pretreated antibody is still able to cause lysis of 48hr old myoblasts, indicating that these cells have antigens unique to their cell surface when compared to 24hr old myoblasts.

fragments were made from the pretreated antibody by papain digest (P-Fabs). These P-Fabs were then tested to see whether or not they inhibited myoblast aggregation. The expection would be that P-Fabs would not inhibit aggregation, as the antibodies do not recognise 24 hour old myoblasts and therefore would not prevent these cells from aggregating. It would seem likely that the 24 hour old and 48 hour old myoblasts share the same aggregating mechanism.

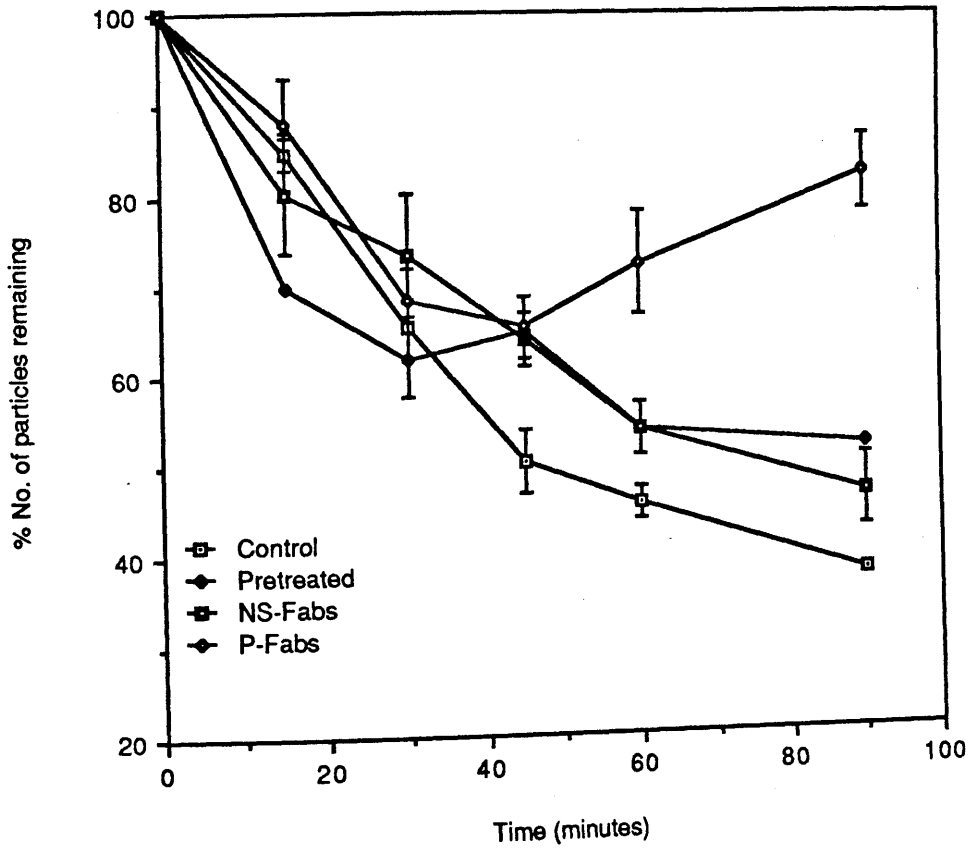
The cells used were 48 hour old myoblasts, and the extent of aggregation was measured by Coulter Counter assay. From Figure 12 it can be seen that P-Fabs have a pronounced effect on aggregation at the final time point (90 minutes). This represents a 71% inhibition of aggregation when compared to the control value, as calculated by the following formula:-

$$\frac{\%\ \text{aggregation (control)} - \%\ \text{aggregation (P-Fabs)}}{\%\ \text{aggregation (control)}} \times 100$$

In the early stages, however, the aggregation shows a similar profile to the control values. Only after 45 minutes does a significant amount of inhibition start to take place. Non-specific rabbit Fabs (NS-Fabs) appear to have a slight inhibitory effect, as does P-ab, in that it slows down the aggregation of the cells, but does not reverse it in the way that P-Fabs do.

Cell death can be discounted as viability was tested by nigrosin staining and found to be over 90%. Aliquots of cells were taken at the final time point (90 minutes), filtered onto 0.22um filter paper, and stained with eosin to give visual

Figure 12:- Inhibition of myoblast aggregation by P-Fabs.




Aggregation is measured by the decrease in number of particles as measured by a Coulter counter. P-Fabs cause a 71% inhibition of aggregation after 90 minutes as compared to the control value.

Key:-

| | |
|---|---|
| Control- | no extra additions. |
| Pretreated- | addition of P-ab (400ug/ml). |
| NS-Fabs- | addition of monovalent antibodies made from non-specific rabbit IgG (400ug/ml). |
| P-Fabs- | addition of monovalent antibodies made from P-ab (400ug/ml) |

confirmation of the reduced number of aggregates in the presence of P-Fabs as compared to the controls.

Discussion.

The inhibition of 48 hour old myoblast aggregation by P-Fabs was an unexpected result. Clearly, P-Fabs will also prevent fusion, as adhesion is a prerequisite for fusion. From Figure 12 it can be seen that the cells are capable of aggregating and do so during the first 45 minutes. After this time aggregation stops and the aggregates start to disperse. Presumably the antigen(s) that P-Fabs recognise is expressed at this time. The subsequent binding of P-Fabs to this antigen(s) then prevents further aggregation, and indeed causes thje aggregates to break up. This tends to suggest that the 48 hour old myoblasts have at least two independent adhesion mechanisms. Furthermore it suggests that when the second adhesion mechanism is expressed, the first adhesion mechanism is lost. If both mechanisms were expressed at the same time then one would expect P-Fabs to have no effect. Once the first adhesion mechanism is switched off, there is then an increased in the number of single cells present. A possible mechanism for the inhibition of aggregation by P-Fabs is shown in Figure 13.

This observation is in agreement with that of Knudsen and Horowitz (1977). Using fusion competent cells, they found that during an aggregation study that cells which had been allowed to aggregate for up to 20 minutes could easily be dispersed with EDTA. After this time, between 30 and 60 minutes, trypsin had to be used to disperse the aggregates. Later it became impossible to dissociate the aggregates, and by

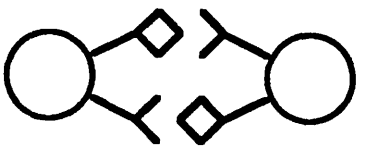
Figure 13:- Possible mechanism for the inhibition of aggregation by P-Fabs.

(a)

Time = 0 minutes.

Newly trypsinised cells. Loss of surface material involved in cell-cell adhesion. Cells are mainly single.

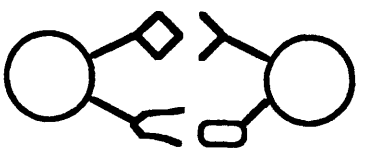
(b)



Time = 15 minutes.

Recovery of cell-surface antigens involved in cell-cell adhesion. The number of single cells decrease.

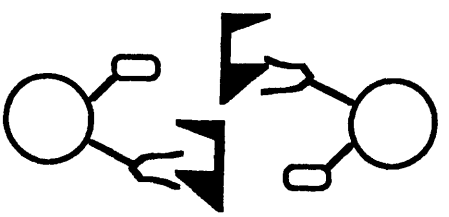
(c)



Time = 30-45 minutes.

The appearance of a secondary adhesion mechanism. It is possible that initially both mechanisms are expressed at the same time.

(d)



Time = 60-90 minutes

The primary adhesion mechanism is replaced by the secondary adhesion mechanism. The antigens involved in this adhesion are recognised by P-Fabs. This results in the breakdown of existing aggregates.

Key

Primary adhesion mechanism.

Secondary adhesion mechanism.

P-Fabs.

2 hours the aggregates had become recognisable multinucleated single cells. They suggested a two-stage adhesion mechanism for fusion competent cells. However the studies of Knudsen and Horowitz (1977) did not involve the use of fusion incompetent cells. It would be interesting to know if the 24 hour old myoblasts displayed the same two-step adhesion. Whether they do or not would not greatly effect the model. If the 24 hour old myoblasts did display a two-step adhesion, then either a tertiary adhesion mechanism would have to be introduced, or only fusion competent cells undergo the loss of the primary adhesion mechanism. However, the time course of events found by Knudsen and Horowitz (1977) fits well with the model, in that the second stage of adhesion is expressed between 30 and 60 minutes.

A dual adhesion system for chick myoblasts has also been found by Gibralter and Turner (1985). Using a protocol similar to that of Takeichi (1977) they found two-independent, non-complementing adhesion systems, distinguishable by their differential susceptibility to proteolysis and dependence on calcium ions for function. It is possible to dissociate chick myoblasts so that only one, both or neither of these adhesive systems is preserved in a functional form.

If the cells are treated with trypsin in the presence of calcium ions, then the calcium-independent system is destroyed, leaving the calcium-dependent system intact. (These cells are designated TC-cells - dissociated with 100ug/ml trypsin and 2.5mM $Ca^{2+}$.) Cells dissociated with 2.5mM EDTA (designated E cells) were found to aggregate in much the same manner in both the presence and absence of

calcium ions. Therefore the activity of the calcium-independent system is thought to control the adhesive interaction of E-cells. Cells treated with both 100ug/ml trypsin and 2.5mM EDTA (designated TE-cells) lose components that are essential to both adhesive systems.

Using cells dissociated in the way described above, Turner and Gibralter (1985) injected TC-cells and E-cells into rabbits, as well as concentrates of the macromolecular material released from the cell surfaces by these treatments (designated E-supe and TC-supe). In this way Turner and Gibralter (1985) hoped to obtain an Fab' that would inhibit muscle cell aggregation. The most promising of the antibodies turned out to be anti-TC-cell Fab' and anti-E-supe Fab', both of which should be directed against the same components. These antibodies were found to inhibit myoblast aggregation. No aggregation was found to take place over the first 120 minutes. At first glance this inhibition appears to be at variance with the model depicted in Figure 13 where aggregation was found to take place over the first 30 minutes. In fact, the difference can easily be explained by the way in which the cells were prepared before injecting them into the rabbit. Turner and Gibralter (1985) used antibodies raised against TC-cells, which have the calcium-dependent mechanism preserved on their surface. (Once again 48 hour old myoblasts had been used, although they had been treated with trypsin after the first 24 hours in order to reduce fibroblast contamination). Therefore, one would expect the anti-TC-cell Fab' to be directed against the calcium-dependent system. As Knudsen and Horowitz (1977) have shown, the initial adhesion between myoblasts is

EDTA-sensitive. Therefore, anti-TC-cell Fab' is directed against the primary adhesion mechanism, and as such no aggregation would be expected. Whereas the P-Fabs used to inhibit aggregation were derived from IgG made against 48 hour old myoblasts and then subsequently adsorbed against 24 hour old myoblasts. Therefore P-Fabs should not recognise the calcium-dependent system.

It is possible that myoblasts have multiple, independently inhibitable, adhesion / recognition steps prior to fusion (Nameroff and Munar, 1976). Inhibition of any one of these steps could cause inhibition of fusion. Certainly it appears that the fusion competent cells have an unique antigen, or set of antigens, and that this antigen is involved in adhesion in some way. It is quite possible that the appearance of a new antigen on the surface of fusion competent cells may result in a more intimate contact between these cells, and lead ultimately to fusion.

# CHAPTER 6:- General Discussion.

The work presented in this thesis concerns the acquisition of fusion competence by myoblasts. Of particular interest was the role of cell surface glycoproteins in mediating the specificity of fusion shown by myoblasts (Okazaki and Holtzer, 1965; Holtzer and Bischoff, 1970). The fact that myoblasts change their cell surface components as they obtain fusion competence has been shown by two independent means. Firstly, lectin-binding to polyacrylamide gels identified a number of changes taking place within the 24hr period prior to fusion. Secondly, antibodies were raised that identified 48hr old myoblasts but not 24hr old ones. Although it has been particularly easy to distinguish differences between 24hr old and 48hr old myoblasts, it has been difficult to identify the antigens involved in the adhesion/recognition phase of myogenesis.

The rather general approach, such as that taken in Chapter 3 (where changes in mannose-containing glycoproteins were investigated), is perhaps a naive one. The fact that certain changes have been identified as the cell moves from fusion incompetence to fusion competence does not necessarily mean that these changes are directly associated with the ability to fuse. It is of little value listing a whole range of changes without designating some function to them. Also, the changes observed are merely a consequence of the method used. In Chapter 3, more than five times as many changes were observed using two-dimensional electrophoresis as compared with one-dimensional electrophoresis. Perhaps an even more sensitive method would reveal even more changes. Pursuing this type of approach would not lead to the identification of the antigens involved in myoblast adhesion/recognition. Much

more specific methods are required. In an attempt to be more specific, a novel application of affinity was employed (as described in Chapter 4). This method used plasma membrane proteins bound to CN-Br activated Sepharose as the affinity matrix. Cell surface proteins, which had been labelled with $^{125}I$ and solubilised in the presence of octyl-glucoside, were passed over the column. After extensive washing with running buffer, the bound proteins were eluted with a salt gradient. Therefore the proteins isolated ought to be cell-surface proteins that are capable of adhering to other cell surface proteins either homophilically or heterophilically. These proteins would then be candidates for those involved in the adhesion/recognition phase of myogenesis.

This method on its own is not sufficient to identify the antigens involved in this phase of myogenesis, but would need to be used in conjunction with other methods (see below). While this novel application of affinity chromatography isolated a number of membrane proteins that might be involved with myoblast adhesion, they could not be isolated in sufficient quantities to test their possible involvement. If the proteins had been available for testing, several approaches could have been adopted. For example, the proteins could have been added to the cell cultures to test their ability to prevent cell adhesion and fusion; the proteins could have been introduced into liposomes to test if adhesion between them could be effected, as well as their ability to bind to myoblasts and myotubes; antibodies could have been raised against them to test whether the resultant Fabs could prevent adhesion and fusion. It would also have been interesting to know whether or not the

antibodies raised against the 48hr old myoblasts (see Chapter 5) were able to recognise the proteins isolated by affinity chromatography.

From the evidence presented in Chapter 3 it is clear that 24hr old myoblasts are different fron 48hr old myoblasts in that they synthesise different sets of glycoproteins. Yet when the adhesion of the cells to cultures of myoblasts and myotubes was compared no significant difference between them could be found (see Chapter 5). However, these cells do behave differently from each other in terms of development. The 48hr old myoblasts go onto fuse with each other in the next 24hrs, whereas the 24hr old myoblasts do not. If fusion is regulated by a specialised adhesion, the fact that there appears to be no difference in the adhesive potential of 24hr and 48hr old myoblasts would suggest that myoblasts display more than one adhesive mechanism. Evidence is presented in Chapter 5 that fusion competent myoblasts might display two independent adhesive mechanisms.

As mentioned earlier, antibodies have proved to be a useful tool in the identification of cell adhesion molecules (see Chapter 1). Antibodies were raised that were able to identify 48hr old myoblasts and not 24hr old myoblasts. It would generally be interpreted that the 48hr old myoblast has an antigen on its cell surface that the 24hr old myoblast does not. However, it is quite possible that the 24hr old myoblast expresses the same protein on its cell surface, but that it is protected from recognition (by the antibody) by another protein. If synthesis of the protective protein is then switched off, the antigen could then become exposed, and be identified

by the antibody. Therefore, it could be the loss of a particular protein that leads to the identification of one cell type from another, and not a gain (as is generally assumed). Some evidence is presented in Chapter 5 for the possibility that myoblast recognition is, in part, regulated by the loss of a particular protein. The Fabs made from the antibodies recognising 48hr old cells were able to inhibit myoblast aggregation, but only after the cells had been aggregating for 45mins. The model (see Chapter 5, Figure 13) suggests that there are two independent adhesion mechanisms, and that both may be expressed at the same time. However, before fusion takes place the primary adhesion mechanism is switched off.

Of the approaches taken, the one most likely to succeed in identifying the antigens involved in the recognition/adhesion phase of myogenesis is the use of antibodies. Although once the Fab-binding antigens have been identified it is still necessary to show that they have an actual function in mediating cell-cell adhesion. With the use of antibodies it might also be possible to dissect the various stages of myogenesis.

Myoblast adhesion is obviously a vey complex affair, and probably involves several stages. Initial contact between myoblasts will occur at the cell periphery. Ultimately, the plasma membranes of each cell must be brought together to allow the cells to fuse. It is likely to be several years yet before this mechanism is fully understood.

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