THE DEVELOPMENT OF A SERUM-FREE MEDIUM FOR USE IN THE CULTURE OF NORMAL AND MALIGNANT HUMAN MELANOCYTES.

by

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Submitted for the degree of
Master of Science.

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May, 1986

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Acknowledgements.

I would like to thank Professor R.M. MacKie for all the assistance and helpful discussion supplied throughout this project. Also Dr. J. Gaukroger for his help with the experimental design and Mrs. I. Campbell for help with staining techniques. Finally thanks to Miss E. Robertson for secretarial services and to my husband who helped with graphics.

Summary.

This project was initiated to study the use of serum-free medium in the culture of human malignant melanoma cell lines and normal human melanocytes. The development of a suitable serum-free medium was carried out in two stages using the two melanoma cell lines.

Firstly, various supplements were tested for their ability to improve cell growth in the basic culture medium (Ham's F-10) devoid of serum. From this work we established a group of five supplements which we termed the standard supplements and these were added at optimal concentrations to all serum-free media examined by us. This group consisted of human transferrin, bovine serum albumin, Intralipid, insulin and B-mercaptoethanol. Various other supplements also proved stimulatory to melanoma cell growth under serum-free conditions, however even in combination with the standard supplements the level of cell growth achieved was only a fraction of that achieved in medium supplemented with serum. Thus we decided to switch to a richer basic medium and found that our requirements were met by medium MCDB104, a modification of Ham's F-10. The standard supplements were re-optimised in this medium and again various potential supplements were tested. Although cell growth, under serum-free conditions, was improved it still did not approach the level achieved in serum-containing medium. Thus it was decided that to improve cell growth under serum-free conditions the basic medium would have to be optimised to suit our requirements.

Thus, the second stage in the serum-free medium development was to re-optimise various components of the MCDB104 medium for the two melanoma cell lines. A batch of MCDB104 deficient in 15 components was prepared. Each of the omitted items was then tested over a concentration range and the optimum concentration for each determined for both cell lines. Using the determined concentrations a "new" MCDB104 medium was prepared for each cell line. Again the standard additives were added.

Although this optimisation improved cell growth, the level achieved was still lower than that obtained with serum supplementation. To achieve a comparable level of cell growth it was felt that all components of the basic growth medium would require to be optimised for each cell line under study.

The development of the serum-free medium was carried out using the two human melanoma cell lines, however it was hoped that any such medium would be useful in the culture of normal human melanocytes. We found that melanocytes would not grow in any of our serum-free media even when supplemented with the routine requirements for melanocyte growth.

We also looked at the possibility of improving upon the two methods we elected to use for the culture of melanocytes. This was done by looking at ways in which the problem of fibroblast contamination and overgrowth could be dealt with and also by looking at factors as potential melanocyte stimulants. We found that calmodulin enhanced melanocyte numbers if used in conjunction with the

phorbol ester and cholera toxin required routinely for melanocyte growth. Fibroblast contamination was not eliminated by any of our methods. Despite this however we were able to culture normal human melanocytes for periods of up to 6 months.

1. INTRODUCTION

For many years cells have been cultured, in vitro, using a complex salt solution (culture medium) supplemented with animal serum, usually bovine in origin. Despite the success of this method, researchers have long since recognised the problems associated with the use of serum. Serum is a highly complex and, as yet undefined, mixture of which many of the components present are poorly characterized and often vary greatly from batch to batch. To overcome these problems, work has been directed towards the elimination of serum supplementation of cell culture medium. This has been undertaken using two different methods.

The first method involves the replacement of serum with various components known to be present in serum and is the result of work carried out by Sato and co-workers (Sato, 1975, Armelin et al, 1974, Barnes and Sato, 1979). They observed that cell lines which were hormone dependent in vivo did not show hormone-dependent growth in vitro unless the serum was depleted of certain hormones before being added to the culture medium. From this they deduced that the serum, before depletion, contained hormones which were stimulatory for cell growth and that the presence of such hormones masked the effect of hormones added directly to the culture medium. Based on this they set about replacing the serum requirement by the addition of a chosen array of hormones at pre-determined concentrations. They also found that other factors present in serum eg transferrin,

bovine serum albumin and serum spreading factor had also to be added to the culture medium. Using this method the specific combination of additives must be determined for each cell line and in most cases the combination is unique to the cell line in question.

The second method is the elimination or reduction of the serum requirement by adjusting the components of the medium as compensation. This is the result of work carried out in the laboratory of Ham(McKeehan and Ham, 1976, McKeehan et al, 1977, Peehl and Ham, 1980, Tsao et al, 1982). To achieve optimal cell growth under these conditions the concentration of serum in the medium is reduced until cell growth is affected. The components of the medium are then adjusted until optimal cell growth is again achieved. This procedure is repeated until either the need for serum is eliminated or the final serum concentration is 1% or less. Again the medium must be specifically adapted for each cell type being studied and again in most cases the adaption is specific for the cell type being investigated.

Our interest in the development of serum-free medium was brought about by on going work on human malignant melanoma. At present the majority of work is centered around the two human melanoma cell lines available to us. They are B0008, derived from a secondary tumour of a 56 year old male caucasian and B00010, derived from a primary tumour of a 53 year old Asian male. Both these cell lines have been fully described by Creasey (Creasey et al, 1979). The fact that very few human melanoma cell

lines are in existance suggests that they are difficult to establish. This is indeed the case and, from our own experience, we have found it very difficult to establish in vitro cell growth from fresh biopsy material. A medium developed specifically for human melanoma cells could make the problem of establishing cell lines easier.

The second, and perhaps more important, reason for our interest in serum-free medium is that studies have shown that the incidence of melanoma in the United Kingdom is 2:1 in favour of females being affected (MacKie et al, 1985). This suggests that hormones or hormone levels may be involved. A cell culture system could be used as a model to look at the effects of hormones on human melanoma, however this cannot be done in serum-supplemented medium as hormones are already present in serum and may mask the effects of hormone additives. Depletion of hormones from the serum may result in sub-optimal cell growth.

Initially, it was decided that rather than try to develope a new serum-free medium those already available in the literature would be investigated. The first medium studied was that developed by Iscove for the growth of lymphocytes (Iscove and Melchers, 1978). This was chosen because of commercial availability. In this medium the serum is replaced by the addition of bovine serum albumin, human transferrin and soybean lipid to the culture medium. The two available cell lines were tested in this medium and the growth profiles obtained were similar to those obtained when the cells were cultured in medium supplemented

with 10% foetal calf serum. However the medium used in this system, Iscove's Modified Dulbecco's Medium, has a high concentration of L-tyrosine (104·20mg/L). This amino acid is involved in melanogenesis during which it oxidises with the enzyme tyrosinase to produce the biochrome melanin. A high concentration of L-tyrosine may lead to increased activity which will, in turn, lead to a build up of toxic by-products in the medium (Graham et al, 1978) which may result in cell damage. For this reason the long term culture of melanoma cells in this medium was not feasible. An alternative serum-free culture system was therefore sought.

A literature search for papers describing a serum-free medium designed for the growth of melanoma cells produced only one paper (Mather and Sato, 1979). The medium described was designed for a cell line derived from the B16 mouse melanoma and the serum requirment was replaced by the addition of hormones and growth factors to the medium. As one of the reasons given for the development of a serum-free medium was to study the effects of hormones on our human melanoma cell lines, this method was rejected.

Although a literature search produced many papers on the growth of cells, mainly lymphocytes, under serum-free conditions eg (Van der Bosch et al, 1981, Brown et al,1983) all were unsuitable. The method described by Brown (Brown et al, 1983) was examined as the basic medium was RPMI 1640 medium in which our cells were routinely cultured.

However this medium proved singularly unsuccessful for human melanoma cells. This was not totally unexpected as reports have suggested that RPM1 1640 medium cannot support cell growth under serum-free conditions without changes to the basic constituents of the medium. Other methods were rejected for reasons including the addition of hormones or a high concentration of L-tyrosine.

However work by Meyskins and Thomson (personal communication) indicated that human melanoma cell lines could be cultured in Nutrient Mixture F-10 (Ham's F-10) (Ham, 1963) supplemented with only bovine serum albumin. In our laboratory this was found to support the growth of the human melanoma cell line B00010 for 4 - 5 days. Ham's F-10 was therefore used as the basic medium for subsequent work. This medium had the advantages of being designed specifically for low serum concentration cell culture and also in having a relatively low concentration of L-tyrosine (table 1). Although short term culture of the melanoma cells was possible in this system, it was obvious that further supplementation would be required for long term culture. The supplements first investigated were those used in the Iscoves medium of which bovine serum albumin was one. However when these were tested at the concentrations recommended by Iscove - human transferrin 1mg/L, soybean lipid 100mg/L and bovine serum albumin 400mg/L - little or no cell growth was observed. Each supplement was then tested over a range of concentrations

Table 1. Concentration of L-tyrosine in various media

Medium	L-tyrosine concentration(mg/L)
Iscoves Modified Dulbecco's	104.20
RPMI 1640	20.00
MEM	36.00
Ham's F-10	1.81

and the optimum concentration for each established. However when each of the supplements was added at the determined concentration the cell growth achieved was only 30 - 40% of that achieved with 10% foetal calf serum supplementation. To improve the medium other substances were tested for growth promoting effects at the recommendation of various authors - insulin (Mather and Sato, 1979, Brown et al, 1983, Darfler and Insel, 1983), B-mercaptoethanol (Brown et al, 1983), sodium selenite (Darfler and Insel, 1983), ferrous chloride and ferrous ammonium sulphate (Iscove and Melchers, 1978, Brown et al, 1983), trace element mixture (McKeehan and Ham, 1976) and putrescine (Ellem and Kay, 1983). An alternative lipid was also sought as the supply of soybean lipid was unreliable and tended to vary greatly from batch to batch - in some cases it was toxic to the cells. The replacement lipid source was found to be a complex lipid mixture, Intralipid, designed for intravenous feeding.

After the testing of the various possible supplements, the basic recipe of Ham's F-10 medium was looked at and was found to have low concentrations of a variety of amino acids. By comparison with the amino acid composition of RPMI 1640 and Iscoves medium, it was noted that most amino acids were

present in Ham's F-10 at lower concentrations. In addition to L-tyrosine, those which varied most were L-cystine, L-isoleucine, L-leucine, L-methionine, L-proline, L-threonine, L-tryptophan and L-valine. With the exception of L-tyrosine, the eight amino acids were tested over a range of concentrations and any affects they had on cell growth were noted. The effects of the amino acids were varied and matters were complicated by the fact that there was evidence to suggest that the effects could be modified by the presence of other components in the medium. The most notable example was the effect of L-cystine added to the medium varied depending on whether or not B-mercaptoethanol was also added to the medium.

To achieve an acceptable level of cell growth in this Ham's F-10 serum-free medium a large number of supplements, including various amino acids, had to be added to the basic medium. This presented some problems the most serious of which was the difficulty in maintaining consistency from batch to batch. Also there was no easy way to determine degradation of the additives. This was especially important in the case of the amino acids as degradation of those already present in the medium had to be taken into account to achieve the optimum concentrations. The large number of supplements to be added also increased the risk of contamination. A further advance was found in medium MCDB 104 (McKeehan et al, 1977) a modification of Ham's F-10. This medium had the same advantages as Ham's F-10 - designed for low serum concentration cell culture and a relatively low L-tyrosine concentration

(5.436 mg/L) - but was found to be a much richer medium than Ham's F-10 and actually contained some of the supplements we added to the Ham's F-10 eg trace element mixture. At the concentrations previously determined, the supplements used by us could be added to MCDB 104 medium and relatively good cell growth was achieved.

All the supplements not already present in MCDB 104 were re-tested and the optimum concentrations re-determined. Although this improved the cell growth obtained with this system, it still did not approach the level obtained with 10% serum supplementation. At this point it was decided that to achieve a level of cell growth comparable with that achieved with 10% serum supplementation a medium would have to be specially designed. There are two distinct approaches to this problem. The first is to add the components known to be present in serum, eg hormones and growth factors, to the basic medium and the second is to alter the concentration of the components in the basic medium to compensate for the lack of serum. As one of our aims was to use the medium to look at the effects of hormones on melanoma cells a modification of the second approach was used.

A relatively good level of cell growth was achieved if MCDB 104 was supplemented with various additives. Thus we decided to use MCDB 104 as the basic medium and alter the constituents of it to our needs. To this end a special batch of MCDB 104 deficient in 16 major components was prepared for us and each of the depleted components was

tested over a range of concentrations and any effect on cell growth noted. Once the concentration of each component was tested and the optimum concentrations determined, the basic supplements - bovine serum albumin, transferrin, Intralipid, insulin and B-mercaptoethanol - were re-tested and their optimum concentrations re-determined.

In addition to studying human melanoma cells we are also interested in growing normal human melanocytes which would provide a vital control for melanoma studies. Human melanocytes are very difficlult to culture. First attempts to culture these cells were made in 1957 (Hu et al, 1957) but the melanocytes lasted for only a few weeks before being overgrown by the other cell types present - fibroblasts and to a lesser extent keratinocytes. In 1982 a new method for culturing normal melanocytes was published (Eisinger and Marko, 1982). This involved the culture of melanocytes, derived from neonatal foreskins, in a medium containing cholera toxin and the phorbol ester, phorbol 12 - myristate - 13 - acetate. Using this medium and a differential trypsinization technique - melanocytes detach from the culture surface before the contaminating fibroblasts -melanocytes can be sustained in culture for periods of up to one year. There are two major problems with is method. The first is that a pure culture of melanocytes is never obtained. Although keratinocyte contamination is eradicated by the presence of the phorbol ester which prevents keratinocyte attachment to the culture surface, fibroblast contamination is merely

controlled by the differential trypsinization technique. The second, and more important problem, is that growth of normal melanocytes by this method requires the presence of phorbol ester in the medium. Phorbol esters are known tumour promoters and the use of them in this culture system brings the "normality" of the melanocytes into question.

Several other workers have published work on the culture of normal melanocytes many require the presence of tumour promoters or carcinogens in the medium. An example is Tsuji and Karasek who have described a method utilizing cholera toxin and isobutylmethylxanthine, a carcinogen, to stimulate cell growth and then adding 5-fluorouracil to the medium to selectively kill the contaminating fibroblasts and keratinocytes (Tsuji and Karasek, 1983). In addition Halaban has increased the yield of melanocytes from the Eisinger method by adding both phorbol ester and isobutylmethylxanthine along with cholera toxin to the medium, then, by the use of genticin, selectively killing the contaminating cell types (Halaban and Alfano, 1984).

More recently Wilkins, Gilchrest and co-workers have described a method for the culture of normal human melanocytes without the use of tumour promoters or carcinogens (Wilkins et al, 1982, Gilchrest et al, 1984, Wilkins et al, 1985). This method involves the purification of a melanocyte stimulating factor from bovine brain and adding this along with epidermal growth factor, nerve growth factor, hydrocortisone, triiodothyronine, transferrin, insulin and cholera toxin to a basic culture

medium (Medium 199). Foetal calf serum at a concentration of 2% is also added at the time of seeding and at each subsequent passage but is omitted at the first medium change. The absence of serum in the cultures for the majority of the time produces cultures which are approximately 95% pure. The removal of the serum is the controlling factor in the removal of contaminating fibroblasts and keratinocytes. Although the keratinocytes are eradicated, the fibroblasts will re-grow if the serum is re-introduced into the system.

Of all the current methods available for the culture of normal melanocytes that of Wilkins, Gilchrest and co-workers is, at present, the most suitable for our project. However, there is room for improvement. The fact that the melanocytes can proliferate in a medium devoid of serum suggests that a serum-free medium would be useful to this system. A defined medium specific for fibroblast culture (McKeehan et al, 1977) and a defined medium specific for the culture of keratinocytes (Peehl and Ham, 1980), both of which are contaminating cell types in melanocyte cultures, have been developed. Thus it would appear possible that a defined, serum-free medium specific for melanocytes could also be developed. It is hoped that the serum-free medium being developed by us for melanoma cells will, with some adaptations, be suitable for normal melanocyte culture.

2. Methods. - Malignant Melanocytes (Melanoma)

The two human melanoma cell lines used, B0008 and B00010, previously described by Creasey (Creasey et al, 1979) were routinely cultured in RPM1 1640 Medium supplemented with 10% foetal calf serum (both Gibco). The basic serum-free medium consisted of either Ham's F-10 or MCDB 104 (both Gibco) supplemented with bovine serum albumin (Gibco or Sigma), human transferrin (Gibco or Sigma), insulin (Wellcome), B-mercaptoethanol (BDH) and Intralipid (Kabivitrum) or soybean lipid (Gibco) at the required concentration. A variety of supplements (table 2) were then tested over a range of concentrations and any effects on the growth of both cell lines noted. This

Table 2. Supplements Tested in The Serum-Free Medium

L-cystine	L-tryptophan	putrescine
L-isoleucine	L-valine	calmodulin
L-leucine	trace element mixture	anti-calmodulin
L-methionine	sodium selenite	cholera toxin
L-proline	ferrous ammonium sulphate	phorbol ester
L-threonine	potassium ferricyanide	methocel

was carried out in the following way :-

The cell lines were grown to confluence in RPM1 1640 supplemented with 2mM L-glutamine and 10% foetal calf serum (all Gibco). The monolayers were then washed with phosphate buffered saline (Oxoid) and then treated with versene. Once all the cells had detached, the resultant suspension was counted using a Coulter counter (Model Dn with co-incidence correction (Coulter Electronics)) and the concentration adjusted as required. The serum-free

medium was made up as outlined above and various dilutions of the component under test were made up in this medium to give a concentration range. The cells were seeded in the appropriate test medium at a concentration of 10^3 or 10^5 cells/test in either 35 x 10mm tissue culture petri dishes or $25cm^2$ tissue culture flasks (Nunc). Controls of the basic medium (ie without any supplements) supplemented with 10% foetal calf serum and the serum-free medium minus the test component were also set up. The cell cultures were incubated at 37^oC in 5% CO_2 for 6 - 7 days. After such time they were harvested and total cell counts made. Results were expressed as either total cell counts or as relative percentages of either of the controls. From these results dose response curves were constructed. The amino acids and trace element mixture were purchased from Gibco. All other test components with the exception of phorbol ester (Consolidated Midland) and Methocel (Fluka) were purchased from Sigma.

A special MCDB 104 medium deficient in calcium chloride, potassium chloride, magnesium sulphate, L-proline, L-tyrosine, disodium hydrogen phosphate, L-glutamine, D or L-valine, sodium pyruvate, folinic acid, niacinamide, thymidine, adenine, choline cholride and inositol was prepared for us by Gibco. A series of defined media with all but one of the omitted items added at the standard concentration were prepared, while the item under test was added over a wide concentration range. Both cell lines were tested and the concentration of each

item optimised. Once these initial optimisation tests were completed, the standard supplements, human transferrin, bovine serum albumin, Intralipid and insulin, were re-tested and their optimum concentrations re-determined.

3. Methods - Cultivation of Normal Human Melanocytes

3:1 Adaption of the Eisinger Method (Eisinger and Marko,1982)

Foreskins were obtained from routine circumcisions and were transported to the laboratory in a sterile universal container (Sterlin). The skin was then washed in phosphate buffered saline (PBS) (Oxoid) plus penicillin-streptomycin and nystatin (both Gibco) at ten times the normal working concentration. The foreskin was then cut into strips of roughly split skin thickness by spreading the skin out, epidermis upward, and lifting up a ridge of tissue with fine forceps. The ridge was then cut using fine dissecting scissors keeping them almost parallel with the surface of the skin (Skerrow and Skerrow, 1983).. The strips were then collected in Earle's Balanced Salt Solution without calcium and magnesium (EBSS w/o Ca + Mg) plus penicillin-streptomycin and fungizone (all Gibco) at five times the normal working concentration and then floated, dermis downward, onto a 0.25% solution of trypsin (Difco) in EBSS w/o Ca + Mg. This was left overnight at 4°C. After approximately 18 hours the trypsin solution was replaced with growth medium (Eagle's minimum essential medium (MEM) with Earle's salts and 25mM HEPES, 0.01mM non essential amino acids, 2mM L-glutamine, 5% myoclone or foetal calf serum, 0.25ug/ml fungizone, 100 units/ml penicillin and 0.1mg/ml streptomycin)(all Gibco) to stop trypsin action. The epidermis was separated from the dermis and the resultant layers from each were separately pooled in EBSS w/o Ca + Mg, shaken vigorously for 30 seconds, then removed from the resultant suspension and

discarded (Skerrow and Skerrow, 1983). The cell suspensions were concentrated by centrifugation at 180g for 10 minutes and the pellet resuspended in growth medium. Cell counts of both preparations were made using a Coulter counter (model Dn with co-incidence correction). 60 x 15mm Primaria petri dishes (Falcon) were seeded with 1.5 x 10^6 cell and growth medium plus 10ng/ml phorbol 12-myristate 13-acetate (PMA) (Consolidated Midland) was added. The cultures were then incubated at 37°C in 5% CO_2 for 24 - 48 hours. After this initial incubation the growth medium was replaced to remove any debris and unattached cells. The cultures were then incubated for a further 48 - 72 hours after which the growth medium was removed, the cells washed with PBS and treated with trypsin (Gibco) to detach the cells. As melanocytes preferentially detach after a short time, this process must be carefully monitored. The detached melanocytes were collected in growth medium, counted and replated at a seeding density of 6 x 10^4 cells/cm^2 in 60 x 15mm Primaria petri dishes, 35 x 10mm Primaria petri dishes or 25cm^2 tissue culture flasks (Nunc). PMA at a concentration of 10ng/ml and cholera toxin (Sigma) at a concentration of 1nM were both added. Using this method we were able to culture human melanocytes for periods of up to six months. Cultures were passaged every 10 - 14 days using the differential trypsinization technique to control the contaminating cell types-fibroblasts and to a much lesser extent keratinocytes.

3:2 Wilkins and Gilchrest Method (Wilkins et al, 1985)

Foreskins were obtained and processed as outlined above. The cell suspensions obtained were then suspended in a growth factor supplemented medium. This consisted of Medium 199 containing 2mM L-glutamine, 0.25ug/ml fungizone, 100 units/ml penicillin and 0.1mg/ml streptomycin (all Gibco) supplemented with 10ng/ml epidermal growth factor, 10^{-9}M triiodothyronine, $5x10^{-5}$M hydrocortisone, 10ug/ml insulin, 10ug/ml transferrin, 100ng/ml 7s nerve growth factor, 10^{-10}M cholera toxin (all Sigma) and 150ng/ml bovine brain extract either prepared by the method of Maciag (Maciag et al, 1979) or commercially obtained (Sigma). The cells were seeded at a density of $5x10^4$ cells/cm^2 in either 60x15mm Primaria petri dishes or 25cm^2 tissue culture flasks. 2% foetal calf serum (Gibco) was also added to the cultures. The cultures were incubated at 37°C in 5% CO_2 for 2 - 3 days. After this initial incubation the cultures were fed every 3 days with the growth factor supplemented medium without the 2% serum additive. After 10 - 14 days, the melanocytes visually demonstrated growth and were apparently free of contaminating cell types (fibroblasts and keratinocytes). At this stage 2% serum supplementation can be continued but if fibroblasts re-appear serum supplementation is discontinued. The melanocyte cultures can be routinely passaged as with other cell types and re-seeded at a density of $5x10^4$/35mm petri dish in growth factor-supplemented medium including 2% foetal calf serum. Using this method melanocyte cultures can be maintained

for up to 6 months.

3:3 Identification of Melanocytes

In tissue culture, melanocytes can be distinguished from keratinocytes on a morphological basis. Under the light microscope melanocytes appear as bipolar or multidendritic cells whereas keratinocytes are rounded or polygonal in shape. After approximately 10 days in culture melanin granules may also be seen in the cultured melanocyte cytoplasm.

Fixed preparations of melanocytes can be stained for the presence of 3,4 -di-hydroxyphenylalanine (DOPA)-positive granules. This DOPA technique is an indicator of the presence of tyrosinase which is found in melanosomes. These are present in melanocytes but not in fibroblasts or keratinocytes.

To show that cultures were not significantly contaminated with fibroblasts, random cultures of every sample were stained for leucine aminopeptidase (Nachlis et al, 1957). This stains fibroblasts but not melanocytes or keratinocytes.

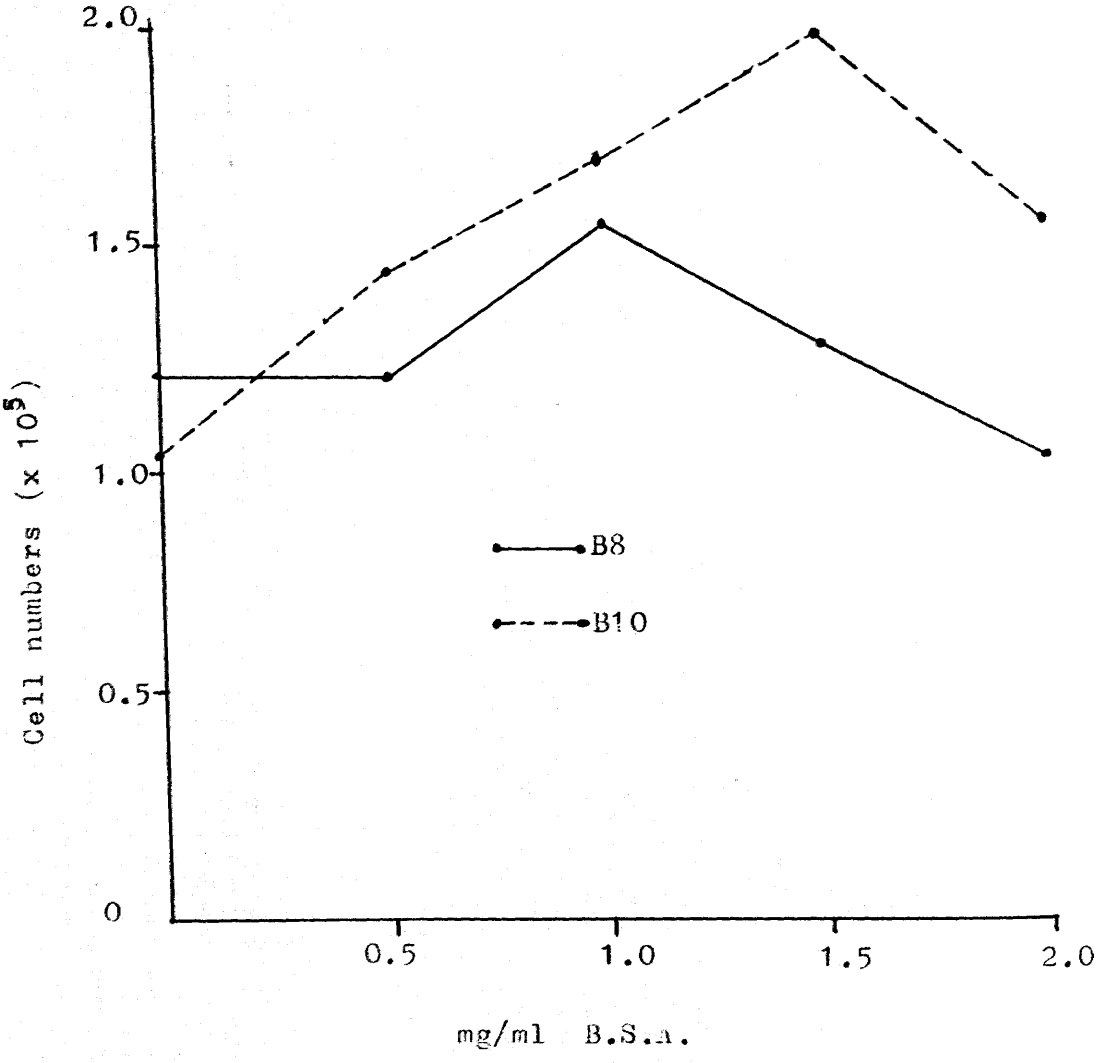


figure 1. Response of B0008 and B00010 cells to bovine serum albumin (B.S.A.)

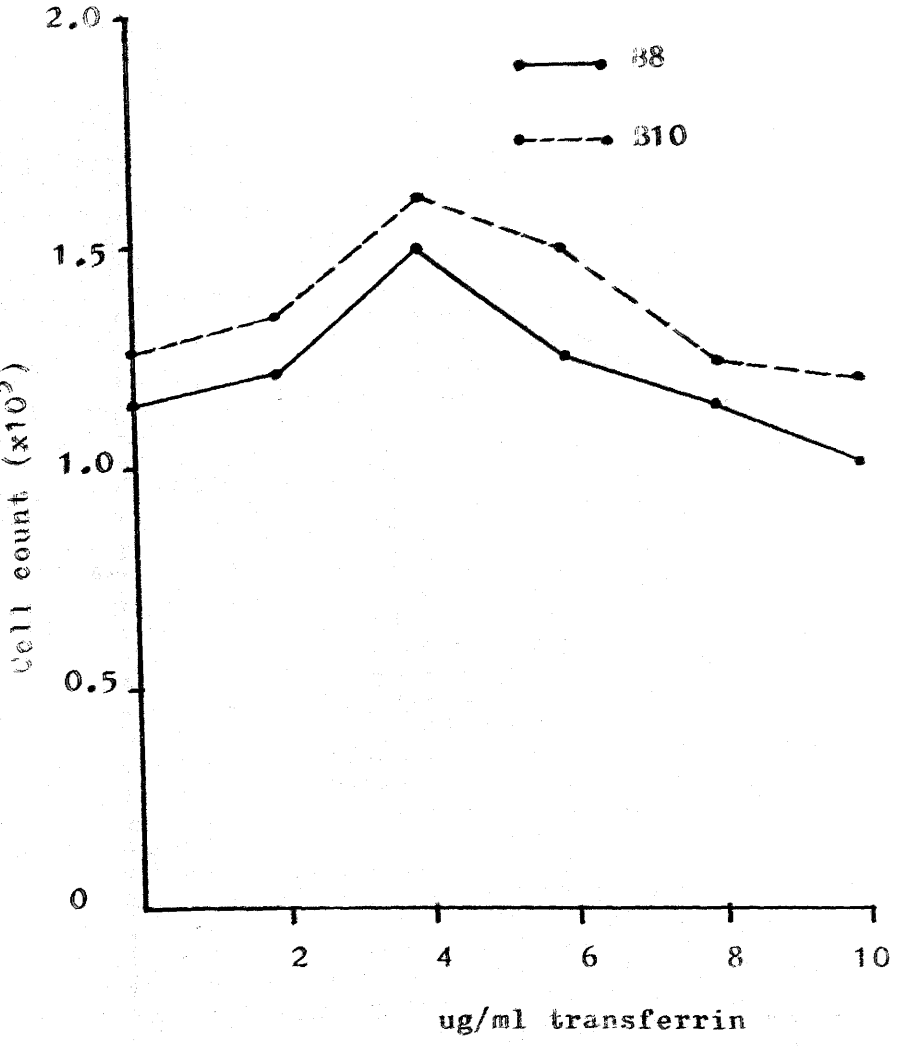


figure 2. Response of B0C08 and B00C10 cells to various concentrations of transferrin.

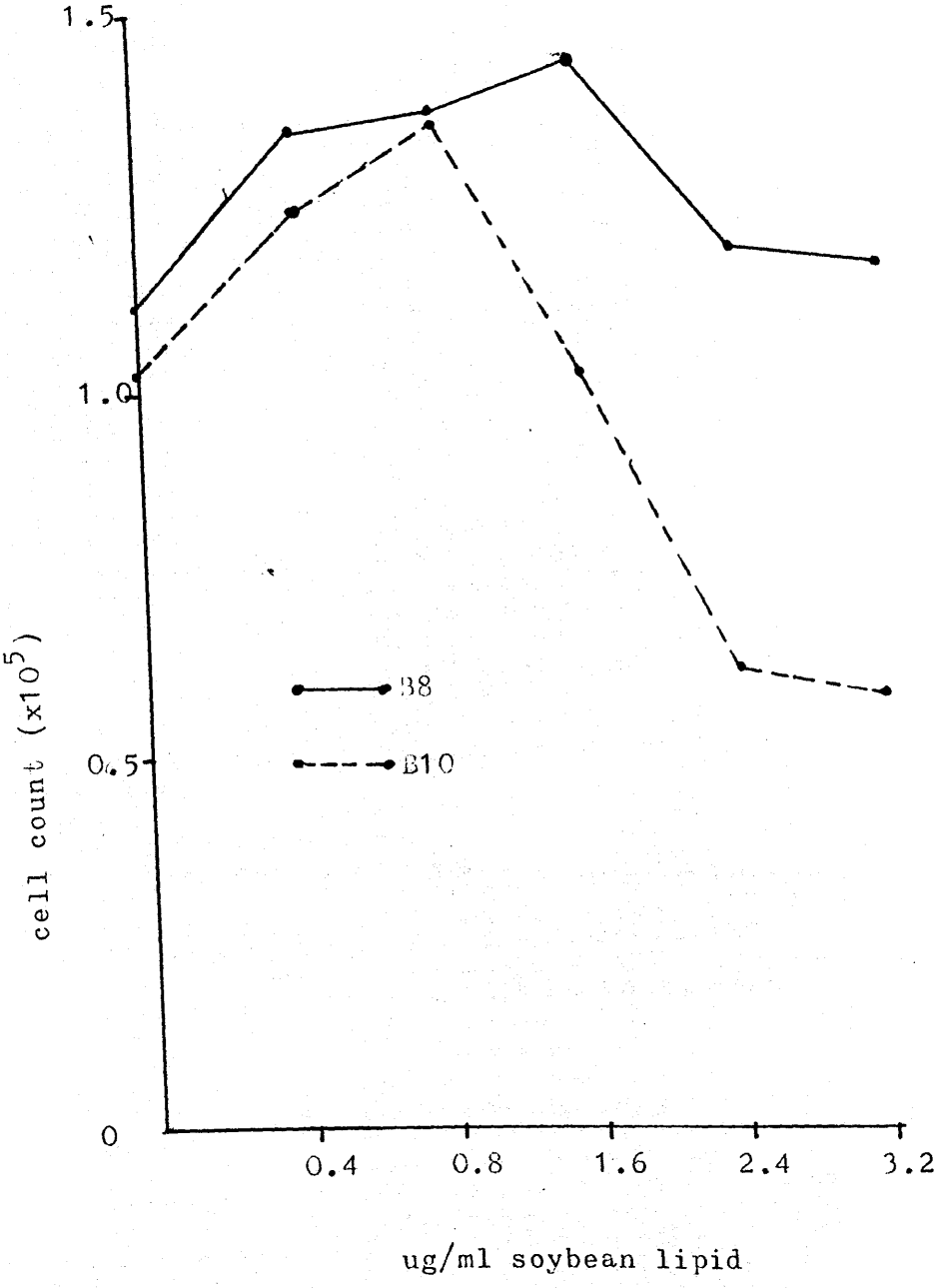


figure 3. Effect of various concentrations of soybean lipid on B0008 and B0010 cells.

4. Medium Optimisation for Malignant Melanocytes (Melanoma) Results.

4:1 Optimisation of Standard Additives

From pilot work it was established that Ham's F-10 supplemented with bovine serum albumin would support the growth of the melanoma cell line B00010 for 4 - 5 days. In an effort to improve on this the other Iscoves supplements (human transferrin and soybean lipid) were added to the medium at the recommended concentrations - 1mg/L and 100mg/L respectively - and bovine serum albumin was added at a concentration of 400mg/L. Supplementation at these concentrations did not support the growth of either cell line. Dose response experiments were set up over a range of concentrations and the optimum concentration of each supplement was established (figures 1-3). The optimum concentration of each supplement is shown in table 3.

Table 3: Optimum concentration of each standard supplement for the melanoma cell lines B0008 and B00010

	Supplement	Concentration
Supplement	B0008	B00010
Human Transferrin	4.0ug/ml	4.0ug/ml
Bovine Serum Albumin	0.10mg/ml	0.15mg/ml
Soybean Lipid	2.4ug/ml	1.6ug/ml

4:2 Replacement of Soybean Lipid with an Alternative Lipid Source

Although the addition of soybean lipid to Ham's F-10, along with the other supplements, supported the growth of both cell lines, there was a problem in that each new batch of soybean lipid had to be assayed against the

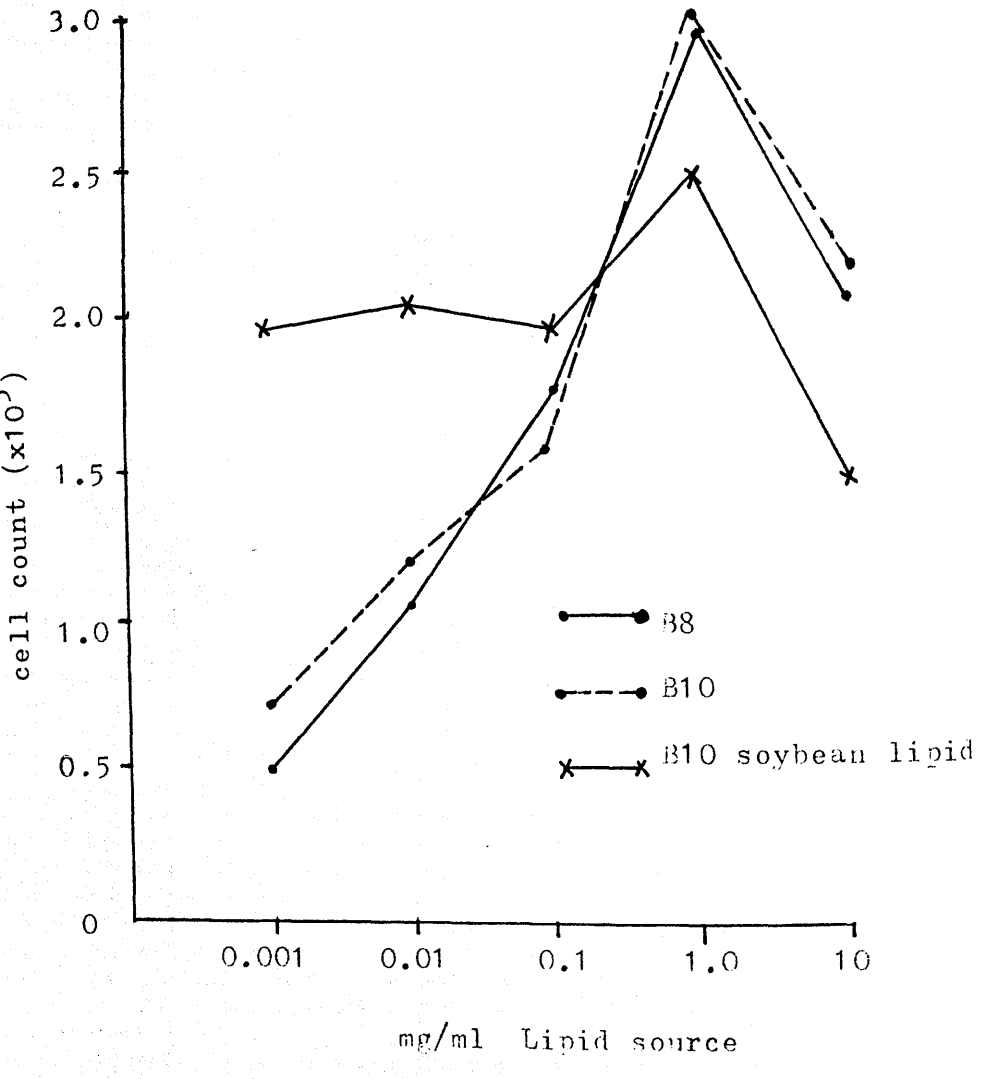


figure 4: Effect of various concentrations of Intralipid on B0008 and B0010 cells. Response of B0010 cells to soybean lipid is also shown.

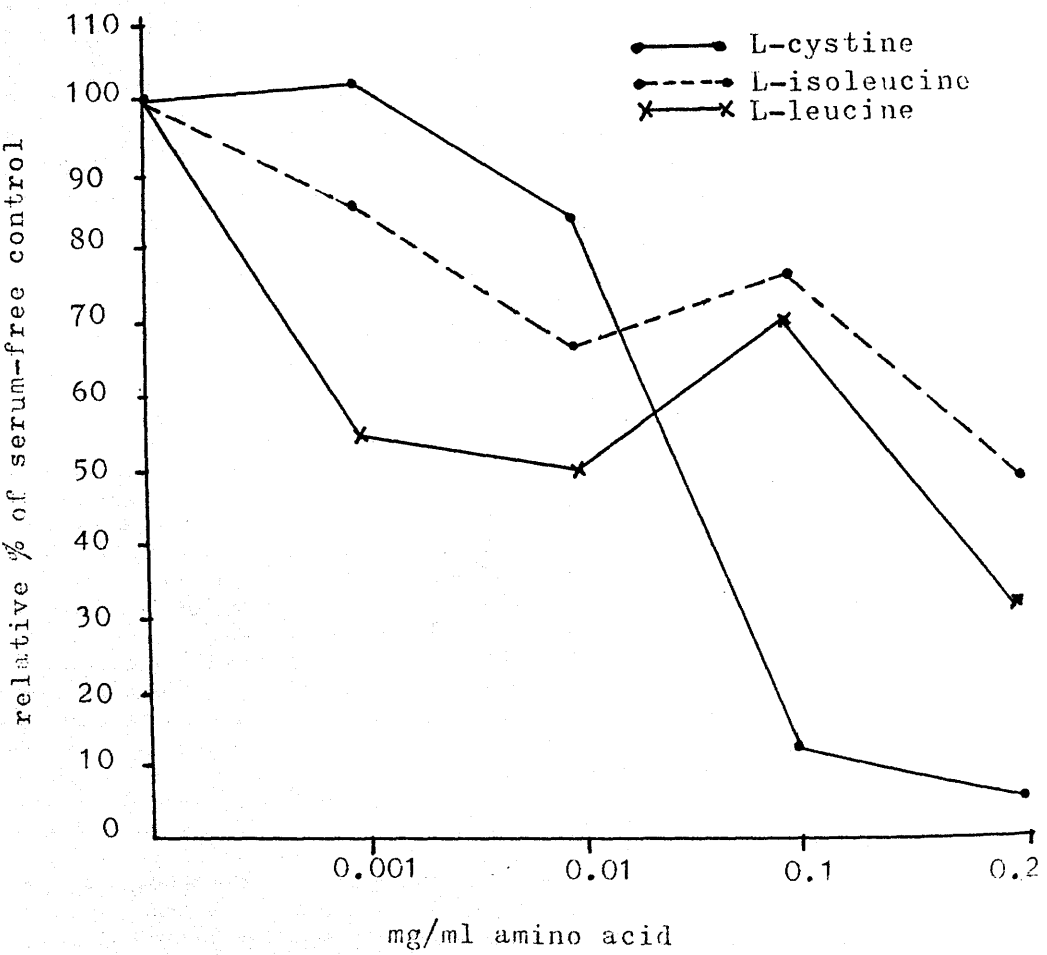


figure 5. The effect of the amino acids L-cystine, L-isoleucine and L-leucine on B00010 cells under serum-free conditions.

previous batch and re-optimised. On several occasions batches obtained had to be rejected as they proved toxic to the cells. Thus an alternative lipid source was sought. The alternative found was the commercially available Intralipid - a complex lipid solution. Using Ham's F-10 supplemented with human transferrin and bovine serum albumin at the concentrations indicated above, Intralipid was tested over a concentration range of 0.001 to 10ug/ml. The results are shown in figure 4. A sample curve obtained with soybean lipid over the same concentration range is also shown (cell line B00010 only). As can be seen the optimum concentration of Intralipid for both cell lines is 1.0ug/ml. At concentrations above 1.0ug/ml, Intralipid cannot be used in cell culture as the suspension of Intralipid in the growth medium becomes very dense and the cells cannot be viewed easily. In an effert to discover the composition of Intralipid, the manufacturers Kabi-vitrum were contacted. However, due to patents they were not prepared to divulge the exact composition of the lipid solution. At present all that we know is that the major constituents are palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid.

Using Intralipid as the new lipid source, human transferrin and bovine serum albumin were re-tested and the optimum concentrations of each re-determined. The optimum concentration for each supplement with respect to both cell lines are shown in table 4.

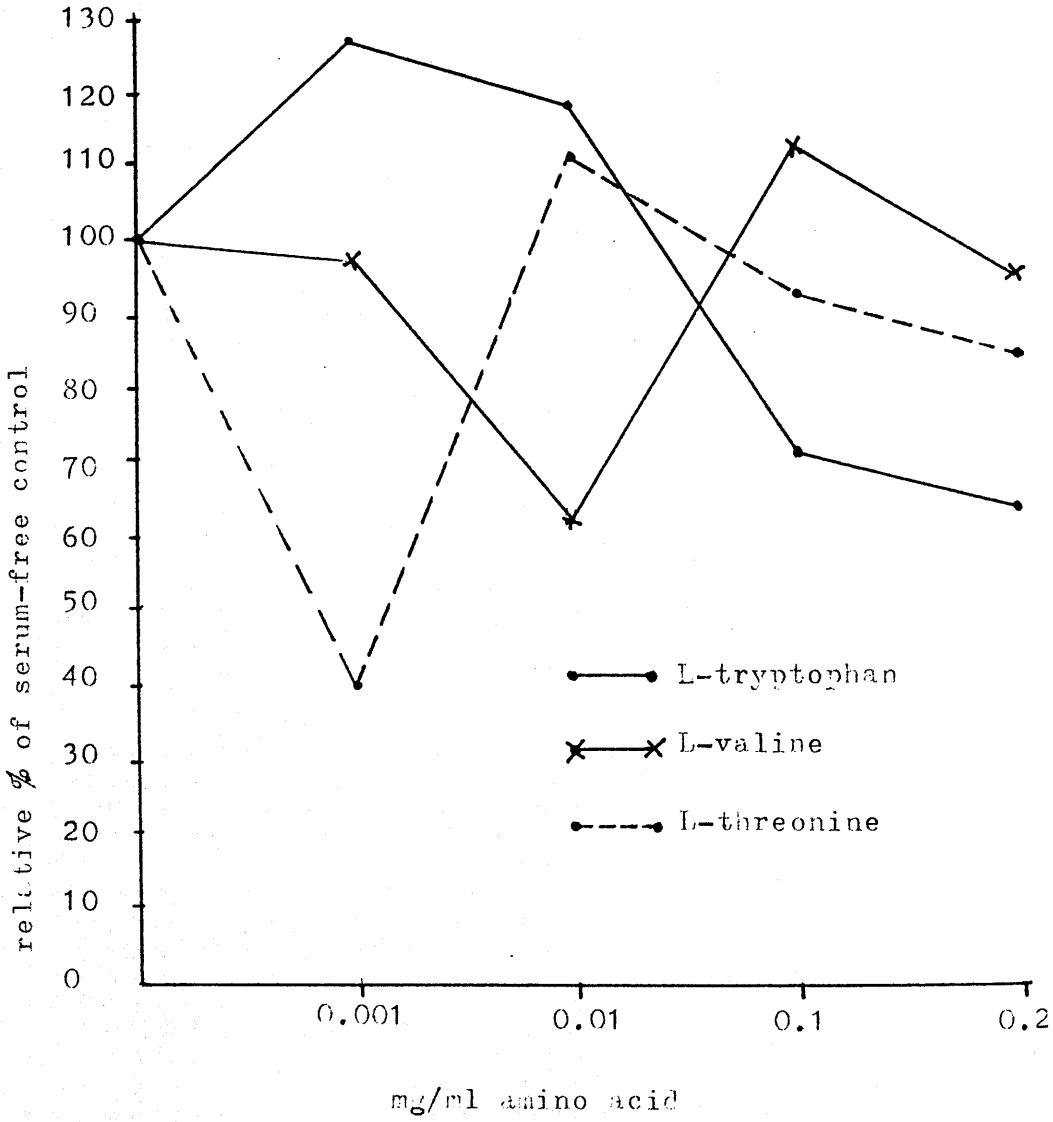


figure 6. The effect of the amino acids L-tryptophan, L-threonine and L-valine on 300010 cells under serum-free conditions.

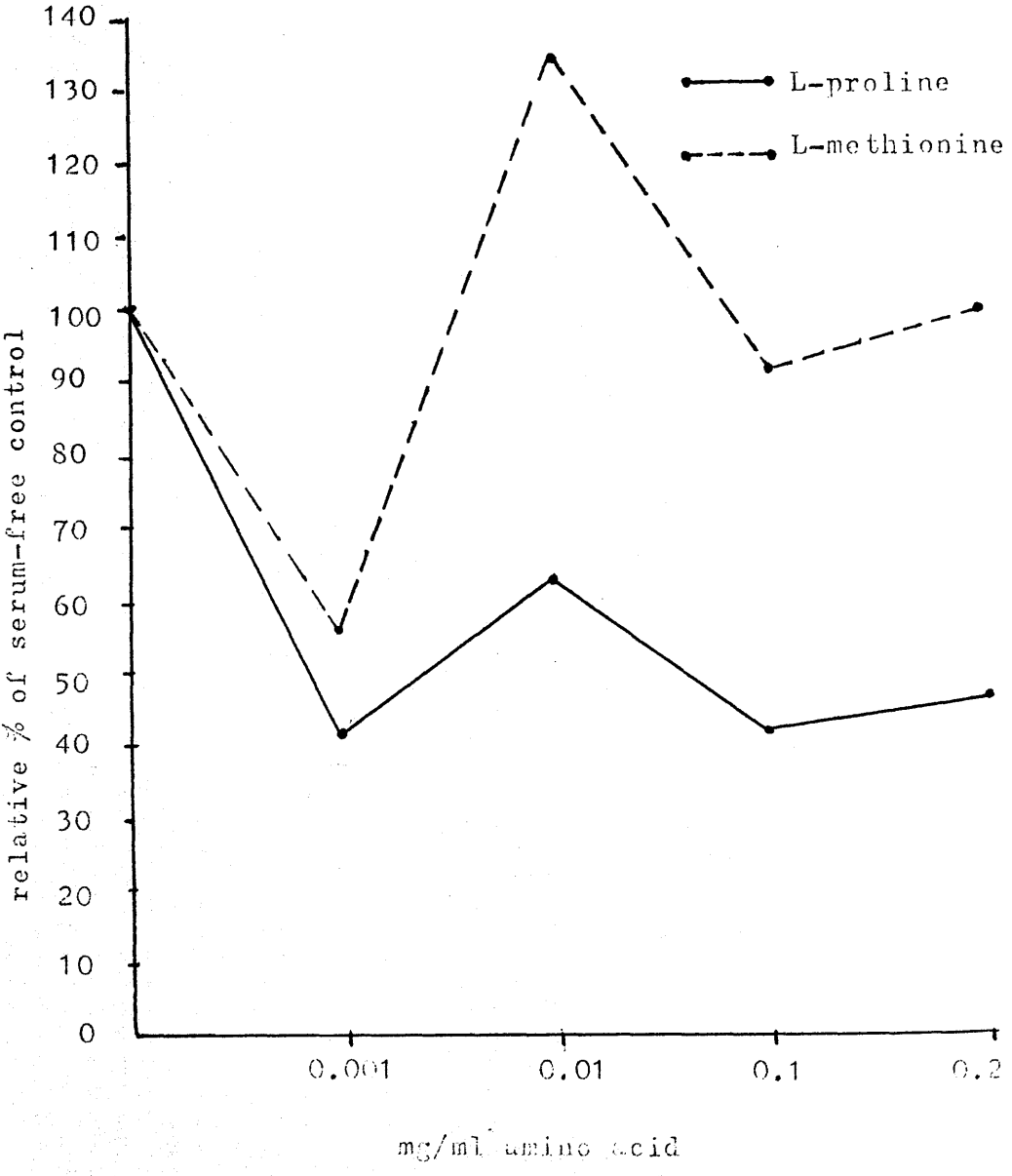


figure 7. The effect of the amino acids L-proline and L-methionine on B00010 cell growth under serum-free conditions.

Table 4: Optimum concentration of each supplement using Intralipid as the lipid source for the melanoma cell lines B0008 and B00010

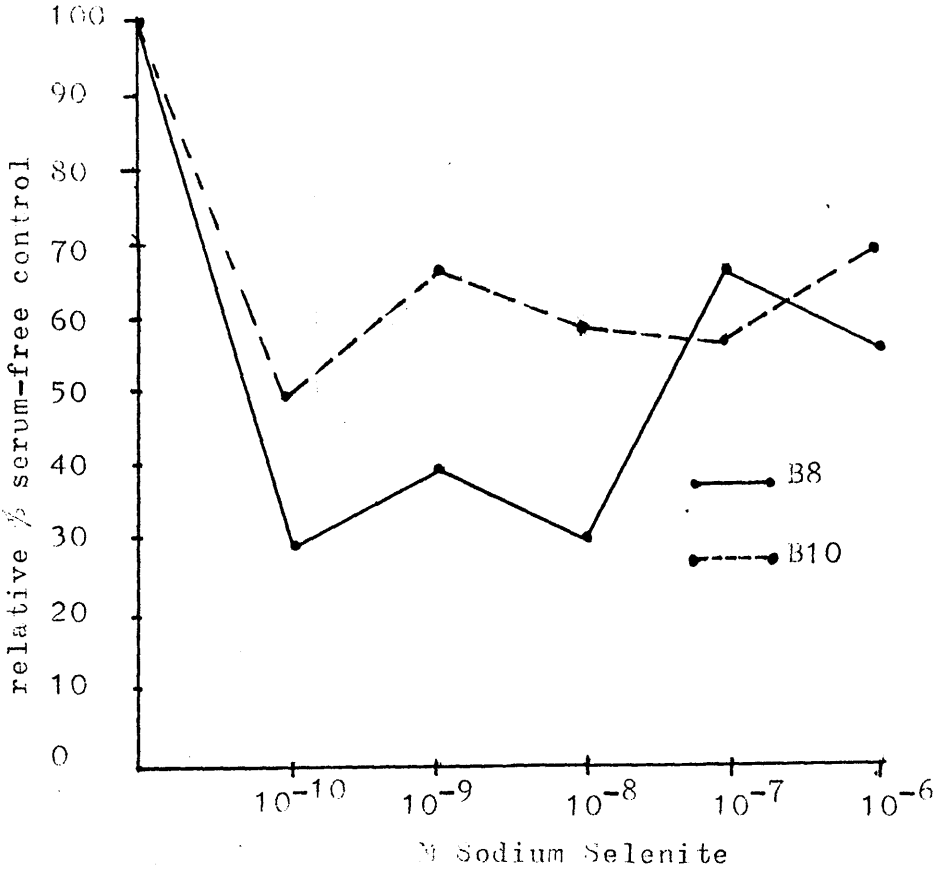
	Supplement Concentration	
Supplement	B0008	B00010
Human Transferrin	4.0ug/ml	4.0ug/ml
Bovine Serum Albumin	0.10mg/ml	0.15mg/ml
Intralipid	1.0ug/ml	1.0ug/ml

4:3 Adjustment of Amino Acid Concentrations

A check on the concentrations of those amino acids present in Ham's F-10 revealed that many of the amino acids were present at much lower concentrations than in RPMI 1640 medium and Iscoves Modified Dulbecco's Medium (table 5). The amino acids which varied most were L-cystine, L-isoleucine, L-leucine, L-methionine, L-proline, L-threonine, L-tryptophan and L-valine. L-tyrosine also varied considerably but for reasons previously outlined no attempt was made to improve cell growth by increasing the concentration of this amino acid. The amino acids were tested over a concentration range of 0.001mg/ml to 0.2mg/ml. The results are shown in figures 5 - 7. The results shown are for cell line B00010 and similar results were obtained using B0008 cells. The results are expressed as a relative percentage of the control set up using the serum-free medium but with no added amino acids.

4:4 Addition of Insulin and B-mercaptoethanol as Standard Supplements.

Insulin was tested as a potential growth stimulator. A dose response experiment was carried out and the optimum


100
90
80
70
60
50
40
30
20
10
0
relative % serum-free control
B8
B10
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
M Sodium Selenite

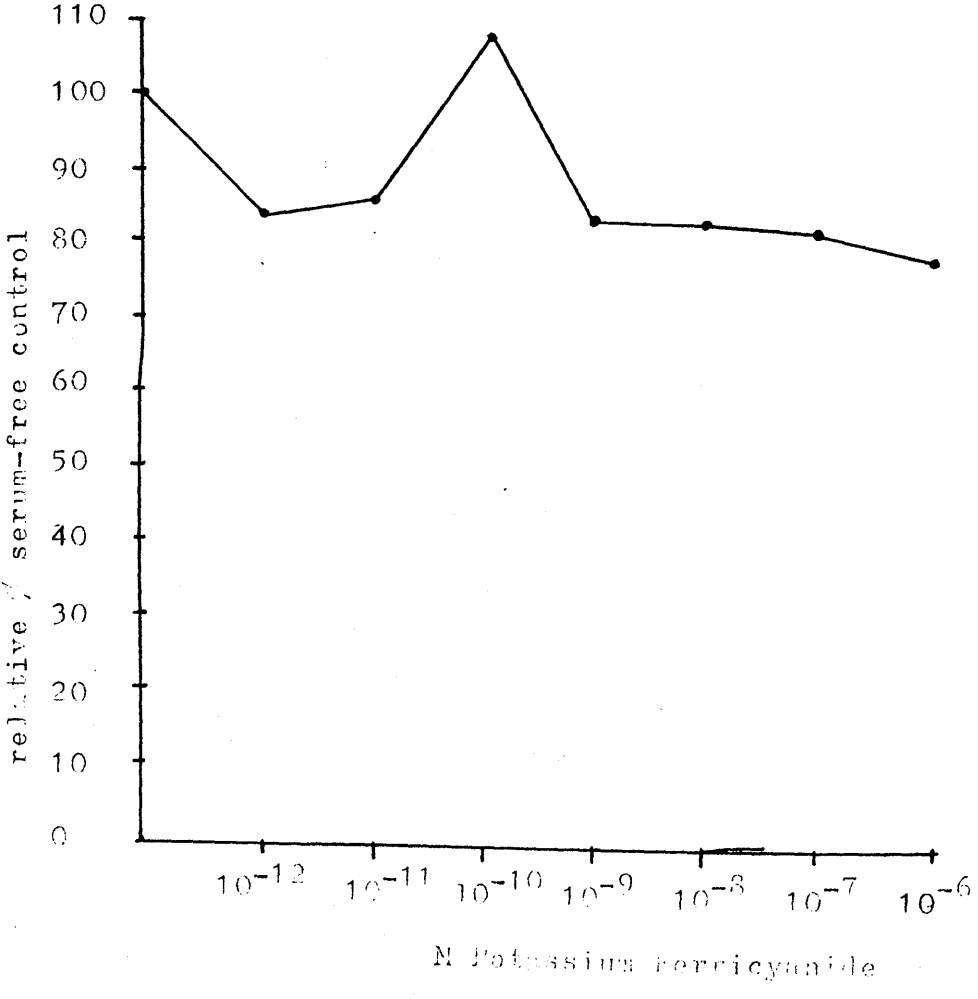

110
100
90
80
70
60
50
40
30
20
10
0
relative % serum-free control
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
M Potassium ferricyanide

figure 8. Effect of sodium selenite on B0008 and B00010 cell growth under serum-free conditions

figure 11. Effect of potassium ferricyanide on B0008 cell growth under serum-free conditions.

concentration of insulin for both cell lines was 0.2IU/ml B-mercaptoethanol was also added to the medium at a concentration of 10^{-7}M. Dose response work indicated that this reducing agent could be added to the medium over a concentration range of 10^{-6} to 10^{-9}M without any noticable effect of the cells. However work by other workers suggests that the optimum concentration of B-mercaptoethanol is 10^{-7}M, thus it was added to our medium at this concentration.

4:5 Supplementation of the Medium with Metal Ions.

Many of the papers published on defined media recommend supplementation with metal ions. Thus sodium selenite, ferrous ammonium sulphate, potassium ferricyanide and a trace element mixture containing manganese, molybdenum, nickel, sodium, tin and vanadium were tested for any effects they may have on the two melanoma cell lines. Sodium selenite was found to be very toxic to both cell lines even at very low concentrations and this is illustrated in figure 8. Ferrous ammonium sulphate and trace element mixture were both stimulatory and the results obtained using these supplements are shown in figures 9 and 10 respectively. Potassium ferricyanide had only a slightly stimulatory effect on BOOO8 cells and this result is shown in figure 11.

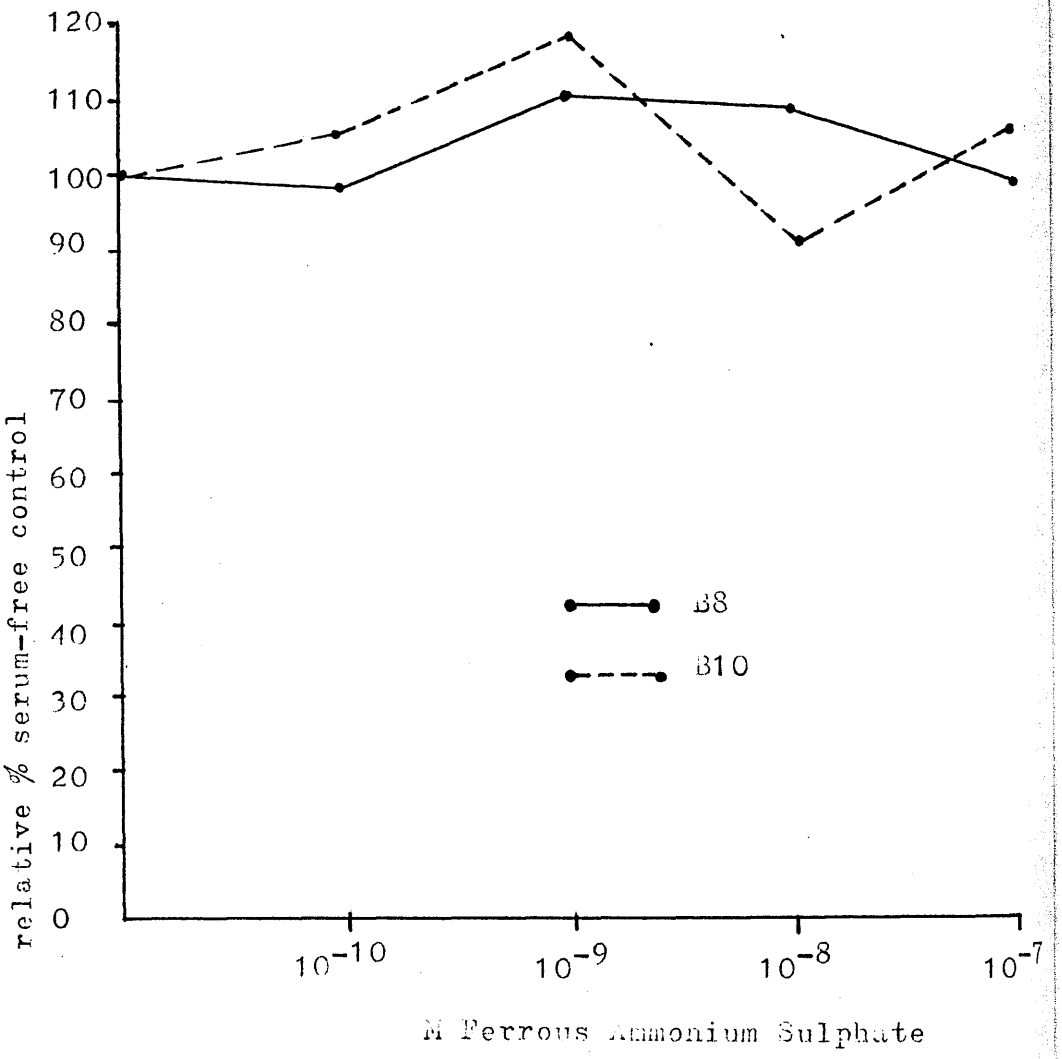


figure 9. Effect of ferrous ammonium sulphate on B0008 and B00010 cell growth under serum-free conditions.

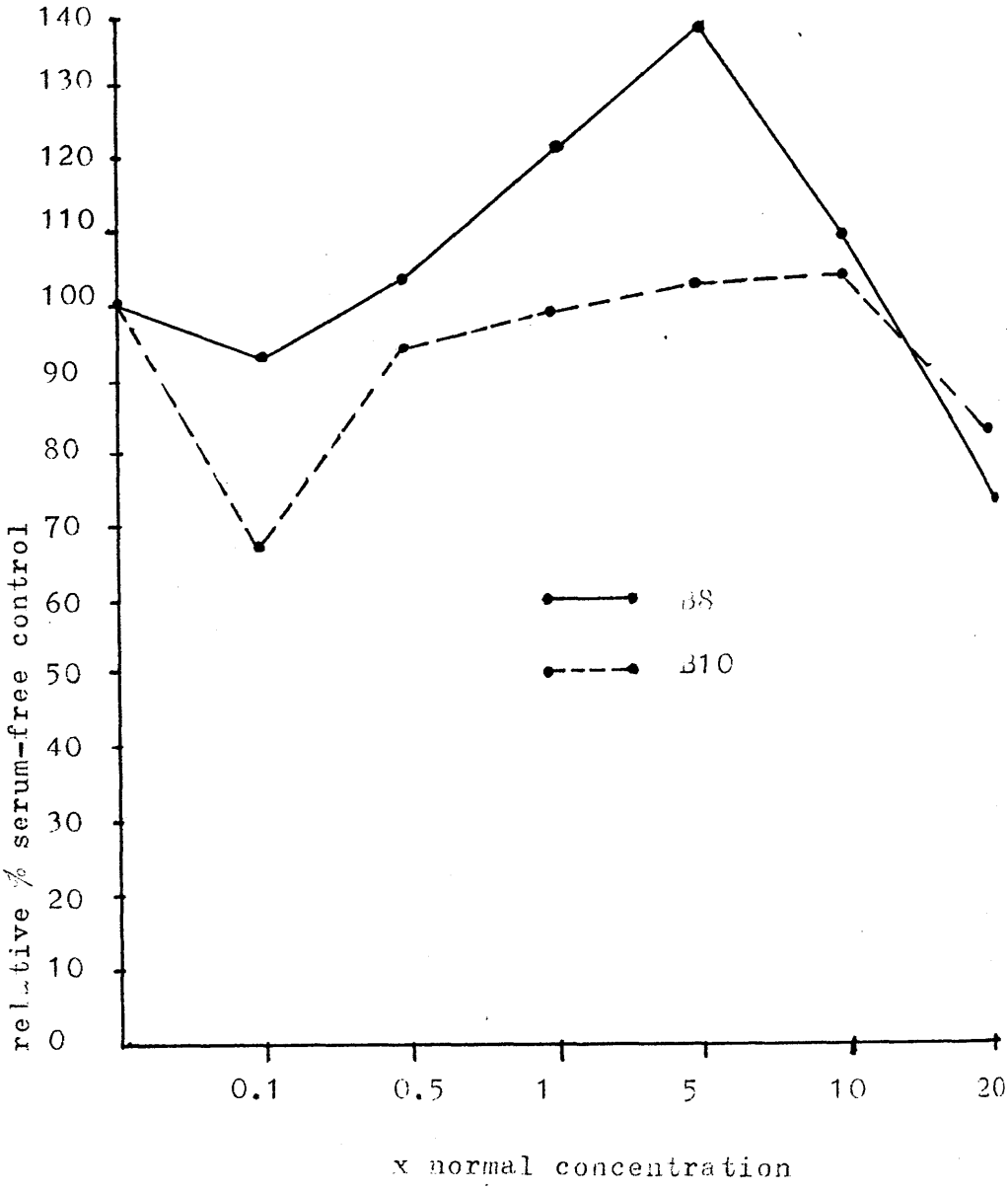


figure 10. Effect of trace element mixture on B0008 and B00010 cells under serum-free conditions.

Table 5: Amino Acid Concentrations in the Media Used in the study

	Amino Acid Concentration (mg/L)			
Amino Acid	Iscove's Medium	RPM1 1640	Ham's F10	MCDB 104
L-Alanine	25.00	-	9.00	8.909
L-arginine	84.00	200.00	211.00	210.77
L-asparagine	28.40	50.00	15.01	15.01
L-aspartic acid	30.00	20.00	13.00	13.31
L-cystine	91.24	50.66	-	-
L-cysteine	-	-	25.00	7.881
L-glutamic acid	75.00	20.00	14.70	14.71
L-glutamine	584.00	300.00	146.00	365.30
glycine	30.00	10.00	7.51	7.507
L-histidine	42.00	15.00	23.00	20.97
L-hydroxyproline	-	20.00	-	-
L-isoleucine	105.00	50.00	2.60	3.939
L-leucine	105.00	50.00	13.00	13.12
L-lysine	146.00	40.00	29.00	36.54
L-methionine	30.00	15.00	4.48	4.476
L-phenylalanine	66.00	15.00	5.00	4.956
L-proline	40.00	20.00	11.50	34.53
L-serine	42.00	30.00	10.50	10.51
L-threonine	95.00	20.00	3.57	11.91
L-tryptophan	16.00	5.00	0.60	2.042
L-tyrosine	104.20	20.00	1.81	5.436
L-valine	94.00	20.00	3.50	11.72

4:6 Interaction of Ferrous Ammonium Sulphate and Transferrin.

As one of the main functions of transferrin is iron transportation we investigated the possibility of enhancing

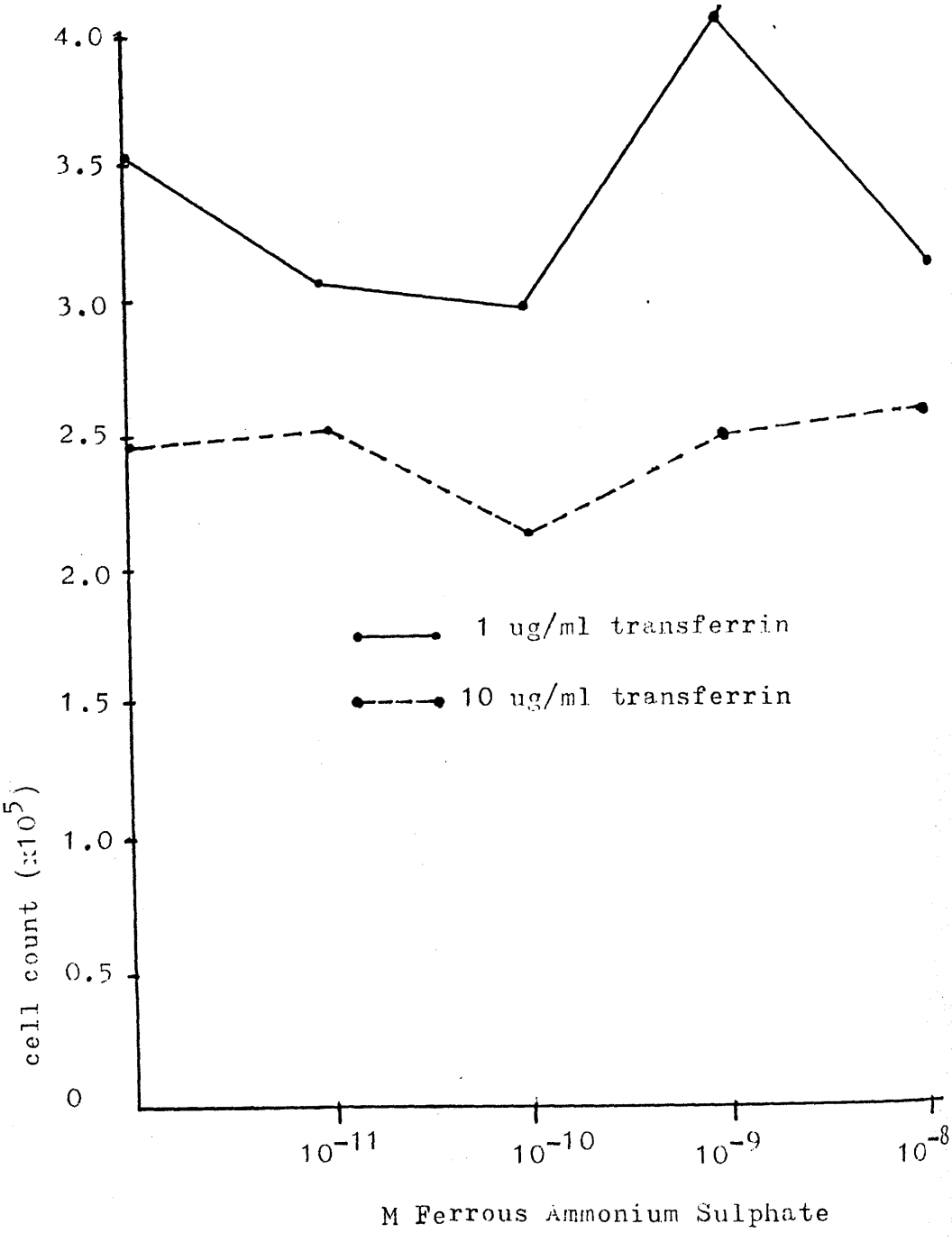


figure 12. The effect of 1 ug/ml and 10 ug/ml transferrin on B00010 cells over a range of ferrous ammonium sulphate concentrations under serum-free conditions.

the stimulatory effect of transferrin by adding excess ferrous ions to the growth medium. We looked at the effect of ferrous ammonium sulphate over a range of concentrations (10^{-8} - 10^{-11}M) using two different concentrations of transferrin - 1ng/ml and 10ng/ml. A similar result was obtained using both cell lines and that for B00010 cells is illustrated in figure 12. A slight increase in cell growth was achieved using a combination of 1ug/ml transferrin and 10^{-9}M ferrous ammonium sulphate. The experiment was repeated using the defined optimum transferrin concentration (4ug/ml) but no significant stimulation occurred (results not shown).

4:7 Stimulation by the Addition of Polyamines

The polyamine putrescine was tested over a wide concentration range (10^{-4}M - 10^{-11}M) on both cell lines. It was found to be slightly stimulatory to both B0008 and B00010 cells at a concentration of 10^{-9}M. The results, expressed as relative percentages of the serum-free control, are shown in figure 13. No other polyamines were tested.

4:8 Interaction of B-mercaptoethanol and L-cystine in the Medium.

Ham's F-10 medium does not contain the amino acid L-cystine. Instead the reduced form L-cysteine is present. When work was being done on the adjustment of the amino acid concentrations (section 4:3. figures 5 - 7) it was noted that the effect of the addition of L-cystine was maximal at the lowest concentration then

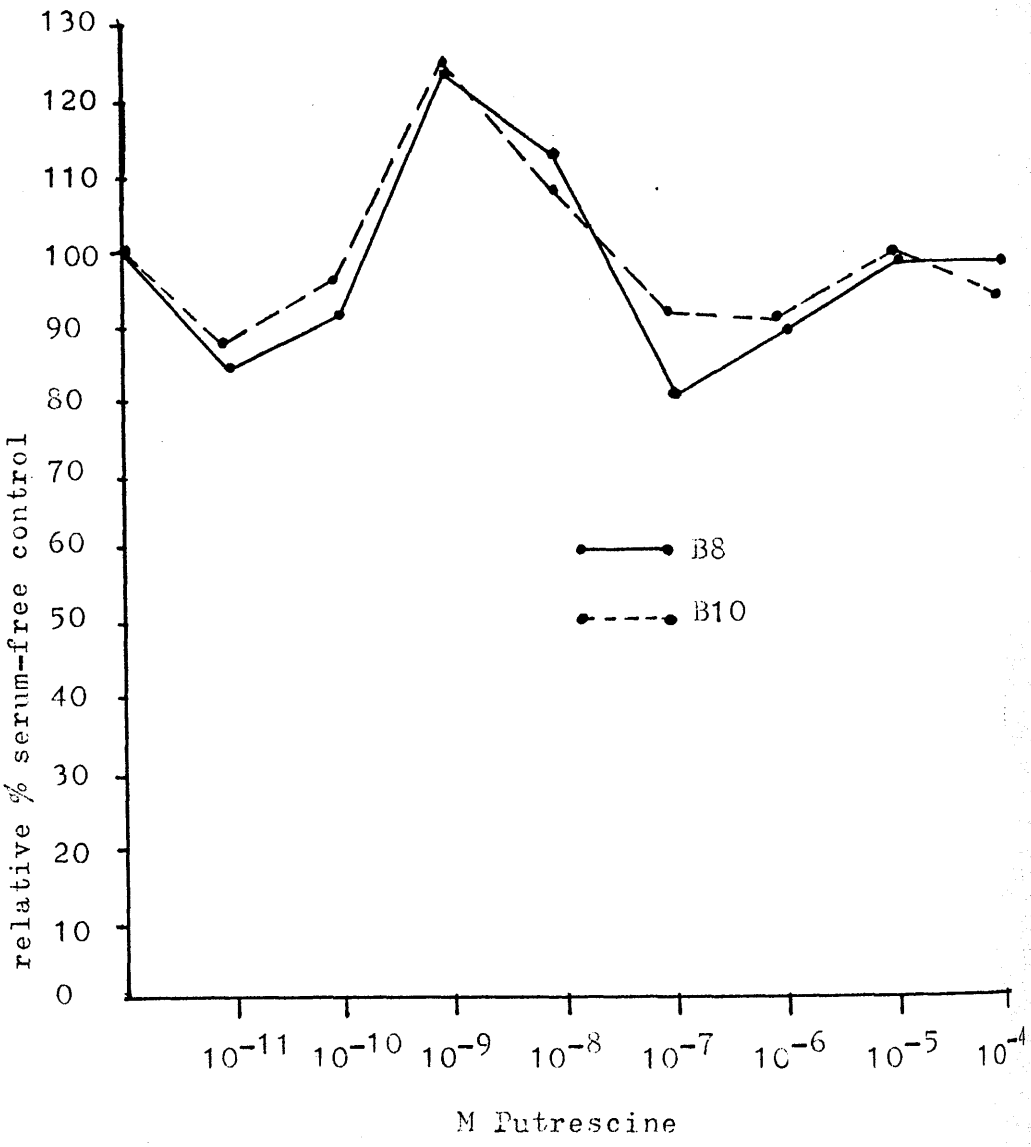


figure 13. Effect of putrescine on B0008 and B00010 cells under serum-free conditions.

tailed off. However, if L-cystine is added and B-mercaptoethanol omitted cell growth is increased. The results are shown in table 6 L-cystine was added at a concentration of 0.005mg/ml and B-mercaptoethanol at 10^{-7}M.

4:9 Growth of Melanoma Cells in Medium Supplemented with Methocel (Methyl Cellulose).

It is known that malignant cells derived from fresh biopsy material can be cultured more successfully if grown in medium containing agar or methylcellulose.

Table 6: The Effect of L-cystine and/or B-mercaptoethanol on Cell Growth.

	Cell Counts(x 10^5)	
Combination	B0008	B00010
w/o L-cystine & w/o B-mercaptoethanol	0.573	0.549
w/L-cystine & w/B-mercaptoethanol	0.779	0.894
w/L-cystine & w/o B-mercaptoethanol	1.228	1.119
w/o L-cystine & w/B-mercaptoethanol	1.195	1.108

Thus we supplemented our serum-free medium with 0.6% methocel. This greatly increased cell growth for both cell lines (table 7) however there were problems. Once the test cultures were removed from the incubator the methocel became very runny and it was difficult to examine the cultures microscopically. Also, when the cultures were harvested they had to be washed several times to ensure that no bits of debris from the methocel remained as this could lead to falsely high counts. In fact, it was difficult to ensure that only cells were counted, thus although improved cell growth was achieved by addition of methocel this practice was discontinued

because of the associated problems.

Table 7: Growth of Melanoma Cells in Methocel Supplemented Medium.

	Cell Count ($\times 10^5$)	
Medium	B0008	B00010
Methocel + Serum-Free Medium	2.787	2.790
Serum-Free Medium	1.377	1.339

4:10 Growth of Melanoma Cells in Ham's F-10 with Additives at Previously Determined Optimum Concentrations.

All the supplements previously tested were added, if they had a positive effect of cell growth, at the determined optimum concentrations. Two different media were made up, the composition of each is shown in table 8, to compare the effects of B-mercaptoethanol and L-cystine. The results are shown in table 9. Two flasks were set up for each cell line in each of the two media. The results are an average of the cell counts obtained from both flasks. The serum-free control consisted of Ham's F-10 supplemented with bovine serum albumin, human transferrin, Intralipid, insulin and B-mercaptoethanol at the optimum concentrations.

Table 8: Composition of Media Using Additives at the Optimum Concentrations

	Concentration			
	Medium A		Medium B	
Component	B00010	B0008	B00010	B0008
BSA*	0.15mg/ml	0.10mg/ml	0.15mg/ml	0.10mg/ml
Intralipid	1.0ug/ml	1.0ug/ml	1.0ug/ml	1.0ug/ml
Transferrin	4.0ug/ml	4.0ug/ml	4.0ug/ml	4.0ug/ml
$(NH_4)_2Fe(SO_4)_2$*	10^{-9}M	10^{-9}M	10^{-9}M	10^{-9}M
Insulin	0.2IU/ml	0.2IU/ml	0.1IU/ml	0.2IU/ml
L-cystine	-	-	0.005mg/ml	0.005mg/ml
L-tryptophan	0.001mg/ml	0.001mg/ml	0.001mg/ml	0.001mg/ml
L-threonine	0.01mg/ml	0.01mg/ml	0.01mg/ml	0.01mg/ml
L-methionine	0.01mg/ml	0.01mg/ml	0.01mg/ml	0.01mg/ml
L-valine	0.1mg/ml	0.1mg/ml	0.1mg3ml	0.1mg/ml
TEM *	10X	5X	10X	5X
Purescine	10^{-9}M	10^{-9}M	10^{-9}M	10^{-9}M
B-mercap.*	10^{-7}M	10^{-7}M	-	-

*(BSA - Bovine Serum Albumin, $(NH_4)_2$ $Fe(SO4)2$ - Ferrous Ammonium Sulphate, TEM - Trace Element Mixture, B-mercap. - B-mercaptoethanol)

4:11 Comparison of Cell Growth in Ham's F-10 and MCDB104

Although the growth of both B0008 and B00010 cells under serum-free conditions was achieved by the supplementation of Ham's F-10 with the various items outlined above, the number of supplements required seemed to continually grow. This fact increased the chances of contamination and also made it difficult to abtain continuity between batches. Thus it was decided that medium MCDB104, a

Table 9: Growth of Melanoma Cells in Medium A and Medium B

	Cell Count ($x10^5$)	
Medium	B0008	B00010
A	2.08	2.215
B	2.10	2.38
Serum-Free Control	1.94	2.01
Serum Control	3.09	3.24

modification of Ham's F-10, would be more suitable to our requirements as it routinely contains trace element mixture and putrescine and has a higher concentration of many amino acids (table 5). It also contains linoleic acid which is thought to be beneficial to cell growth under serum-free conditions. Thus tests were set up to compare the growth of both cell lines in MCDB104 and Ham's F-10. MCDB104 was supplemented with bovine serum albumin, human transferrin, Intralipid, insulin and B-mercaptoethanol at the optimum concentrations determined for each cell line using Ham's F-10, while Ham's F-10 was supplemented as shown in table 8 (medium B). The results are shown in table 10. For both cell lines, MCDB104 improved cell growth under serum-free conditions.

Table 10: Comparison of B0008 and B00010 Cell Growth Under Serum-Free Conditions Using Ham's F-10 and MCDB104

	Cell Count ($x10^5$)	
Medium	B0008	B00010
Ham's F-10	1.166	0.705
MCDB104	3.629	2.749
Serum Control	7.799	6.085

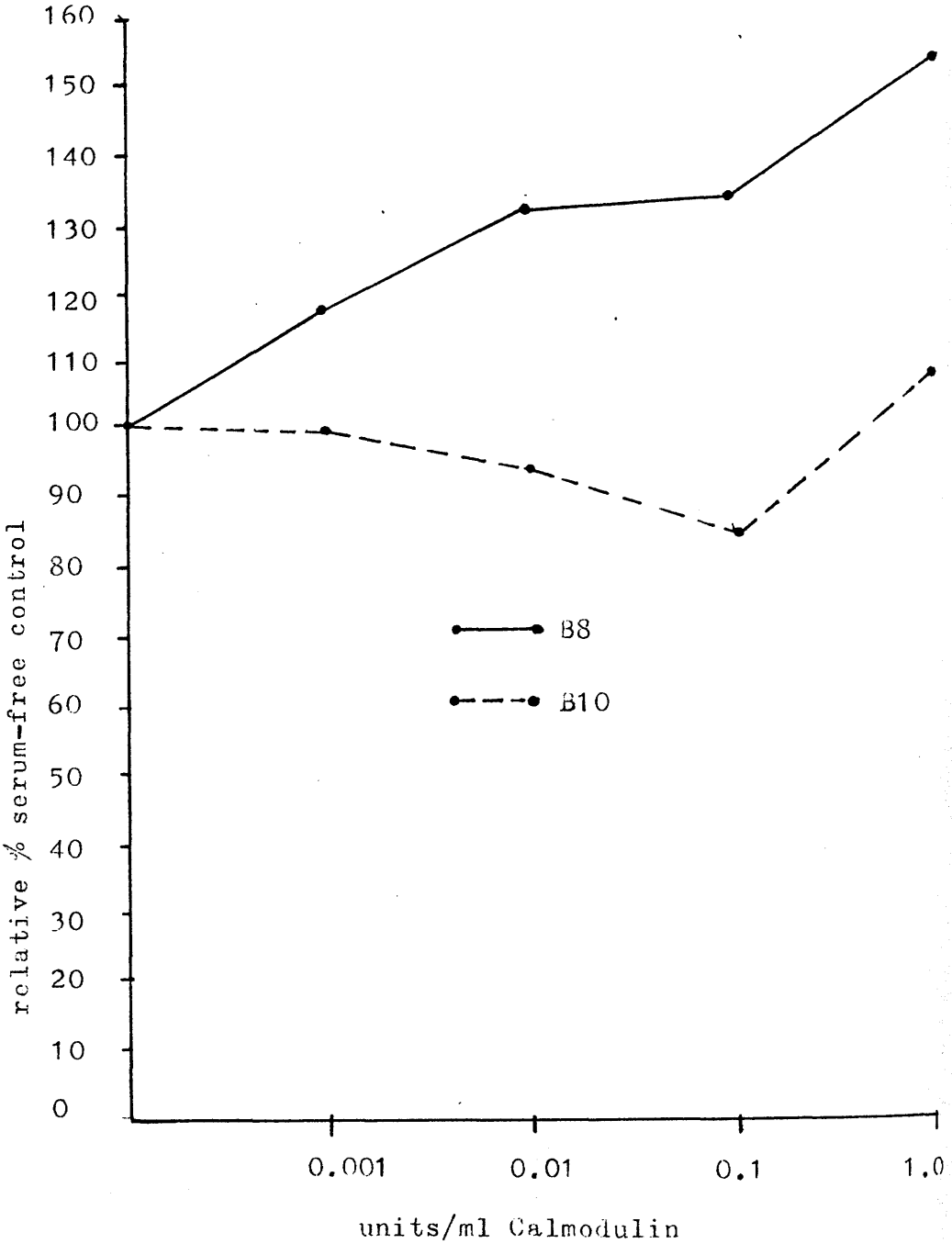


figure 14. The effect of calmodulin on B0008 and B00010 cells under serum-free conditions.

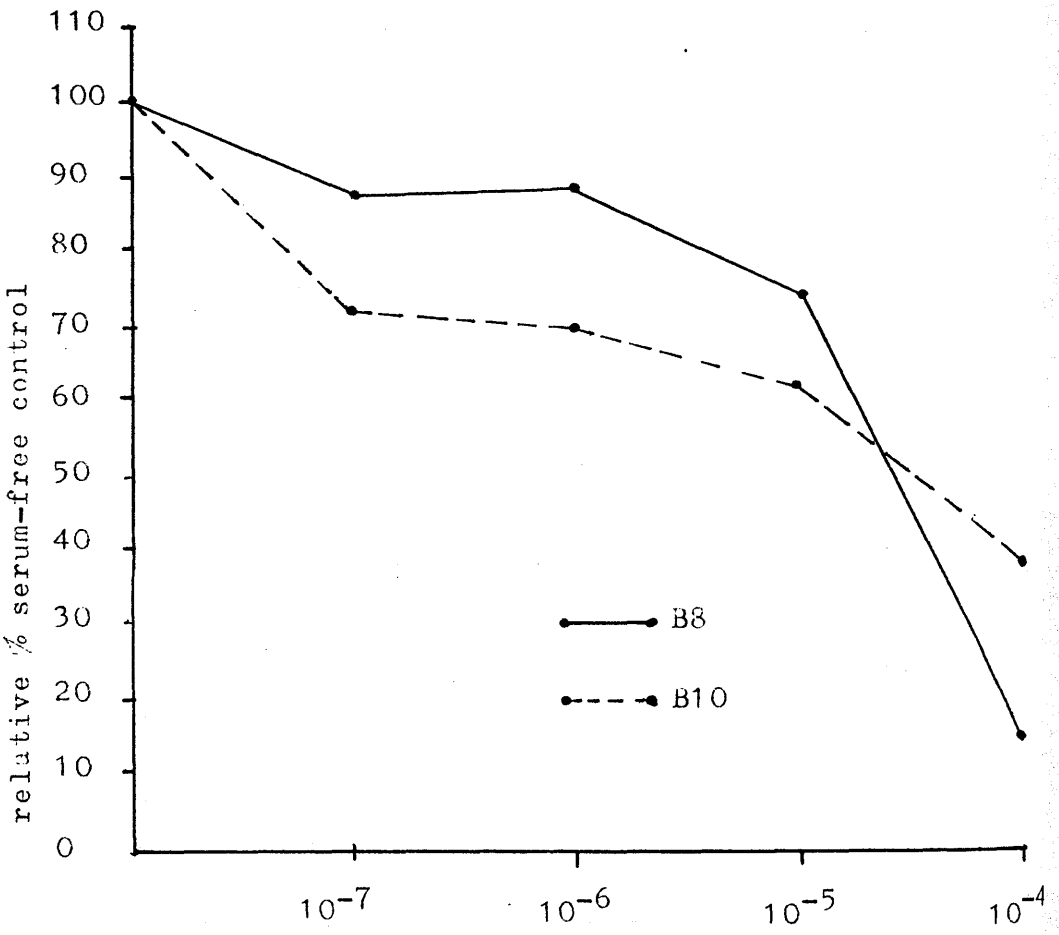


figure 15. The effect of anti-calmodulin on B0008 and B00010 cells under serum-free conditions.

4:12 Re-optimisation of the Standard Supplements in MCDB104

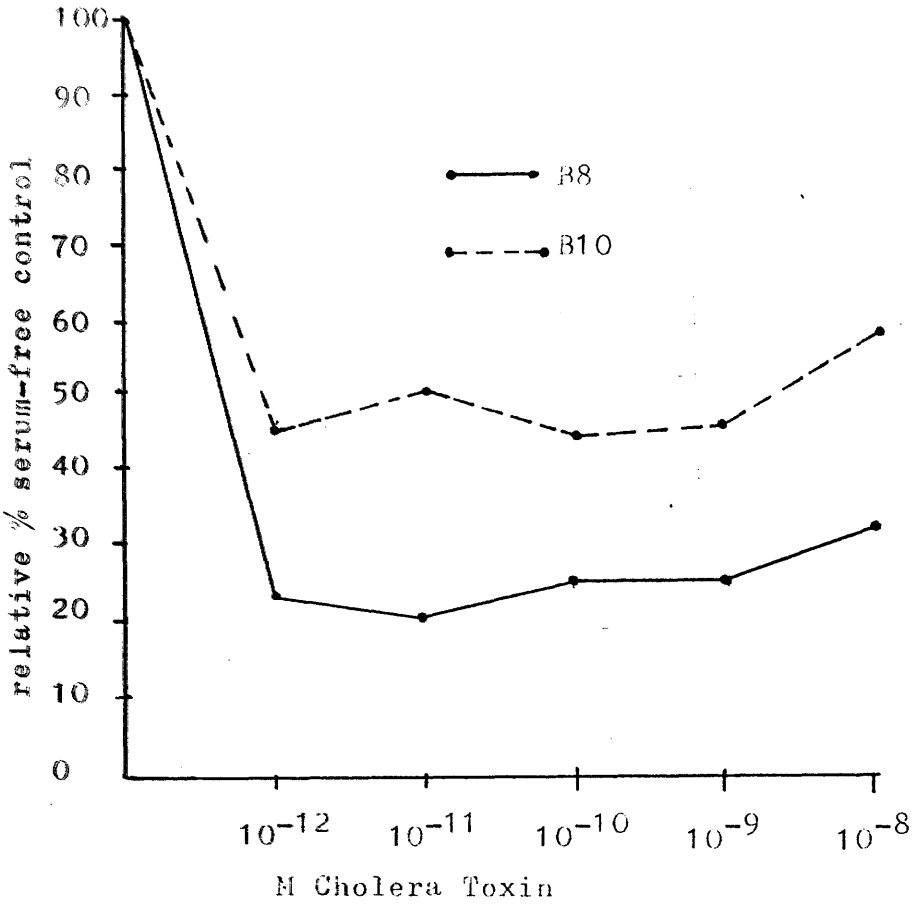
The standard supplements, i.e. bovine serum albumin, human transferrin, Intralipid, insulin and B-mercaptoethanol, which were routinely added to Ham's F-10 serum-free medium were tested again for both cell lines and the optimum concentration of each supplement determined, with the exception of bovine serum albumin, the optimum concentration of each supplement remained the same. These concentrations are shown in table 11.

Table 11: Optimum Concentrations of the Standard Supplements in MCDB104 Medium

	Concentration	
Supplement	B0008	B00010
Bovine Serum Albumin	0.15mg/ml	0.10mg/ml
Human Transferrin	4.0ug/ml	4.0ug/ml
Intralipid	1.0ug/ml	1.0ug/ml
Insulin	0.2IU/ml	0.2IU/ml
B-mercaptoethanol	10^{-7}M	10^{-7}M

4:13 Supplementation of MCDB104 with Calmodulin and Anti-calmodulin

It has been reported that calmodulin like activity is present in foetal calf serum and work in our laboratory has suggested that calmodulin and anti-calmodulin have an effect on human melanoma cells. Thus we added various concentrations of each substance to cultures of both cell lines in serum-free medium. Calmodulin was tested over a range of 0.001unit/ml to 1unit/ml while anti-calmodulin was tested from 10^{-4}M to 10^{-7}M. The results are shown in figures 14 and 15. In general, it can be seen that, overall, calmodulin proved stimulatory to the cells,


100
90
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relative % serum-free control
B8
B10
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
M Cholera Toxin

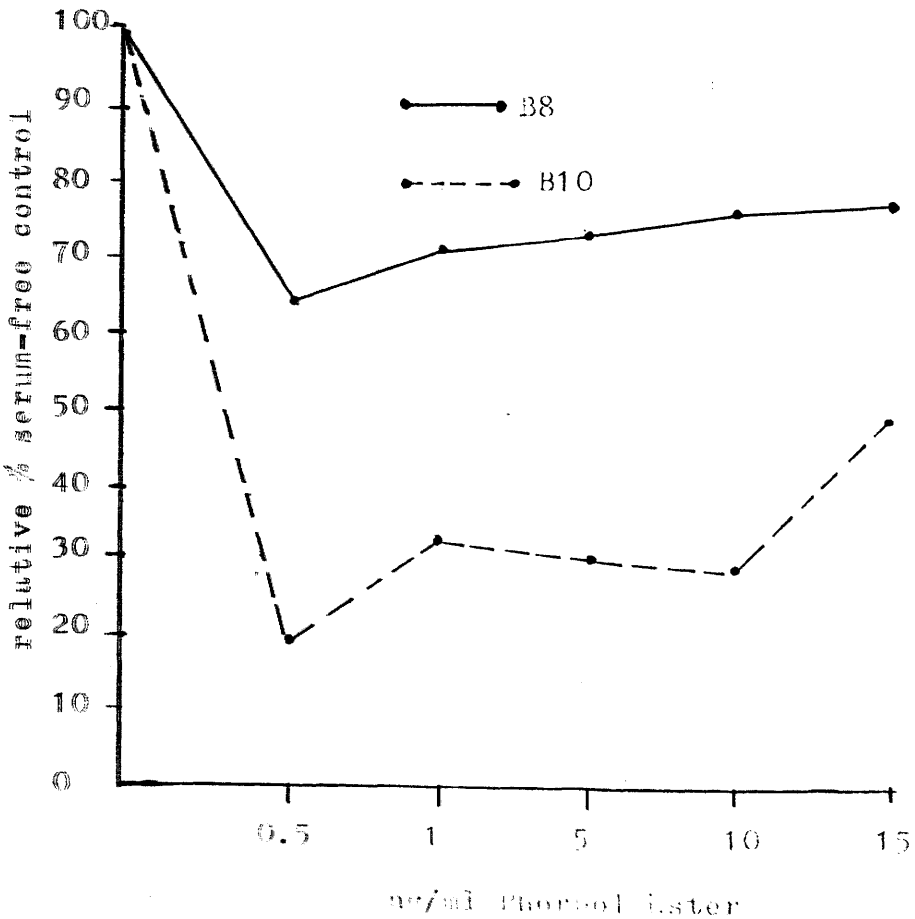

100
90
80
70
60
50
40
30
20
10
0
relative % serum-free control
B8
B10
0.5
1
5
10
15
ng/ml Phorbol Ester

figure 16. The effect of cholera toxin on B0008 and B00010 cells under serum-free conditions.

figure 17. The effect of phorbol ester on B0008 and B00010 cells under serum-free conditions.

especially BOOO8 cells, while anti-calmodulin was inhibitory.

4:14 Supplementation of MCDB104 Serum-Free Medium with Cholera Toxin.

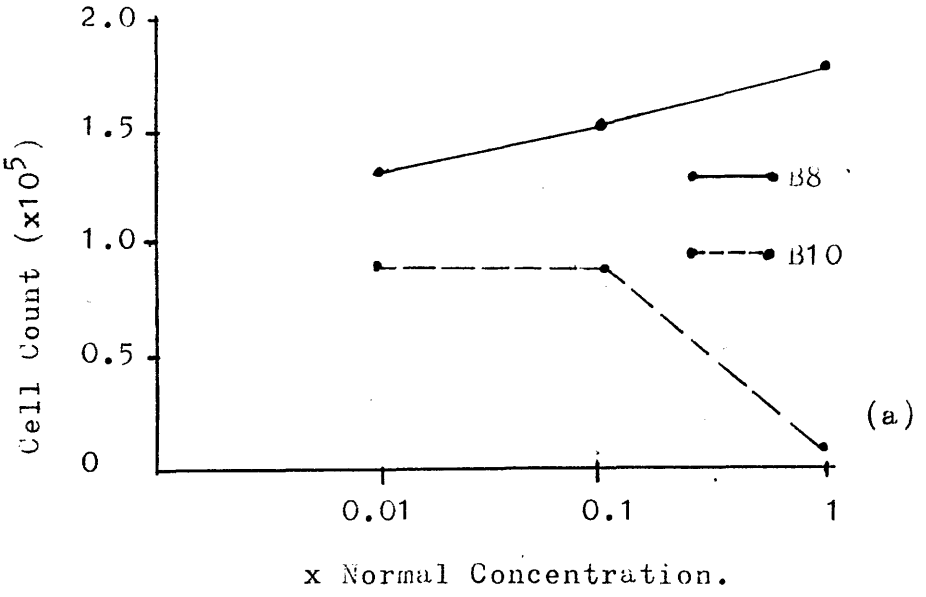
Cholera toxin is used by us to stimulate the proliferation of normal human melanocytes in culture. Thus we tested cholera toxin over a concentration range of 10^{-8}M to 10^{-12}M in serum-free medium for any stimulatory effects on both cell lines. The results are depicted in figure 16 and as can be seen, cholera toxin proved to be inhibitory to the growth of both cell types at all tested concentration.

4:15 Supplementation of MCDB104 Serum-Free Medium with Phorbol Ester.

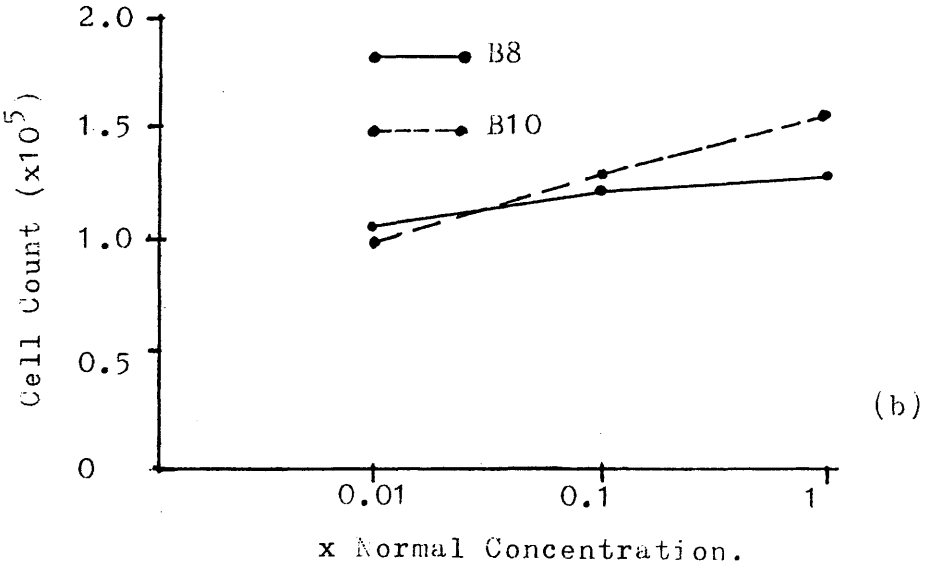
In the culture of normal human melanocytes the phorbol ester phorbol 12-myristate - 13-acetate at a final concentration of 10ng/ml is also added to the growth medium. Thus it was tested for potential growth promoting effects. The concentration range looked at was 0.5-20ng/ml. As with cholera toxin, the phorbol ester proved to be inhibitory at all tested concentrations. The results are shown in figure 17.

4:16 Optimisation of Some of the Components of MCDB104.

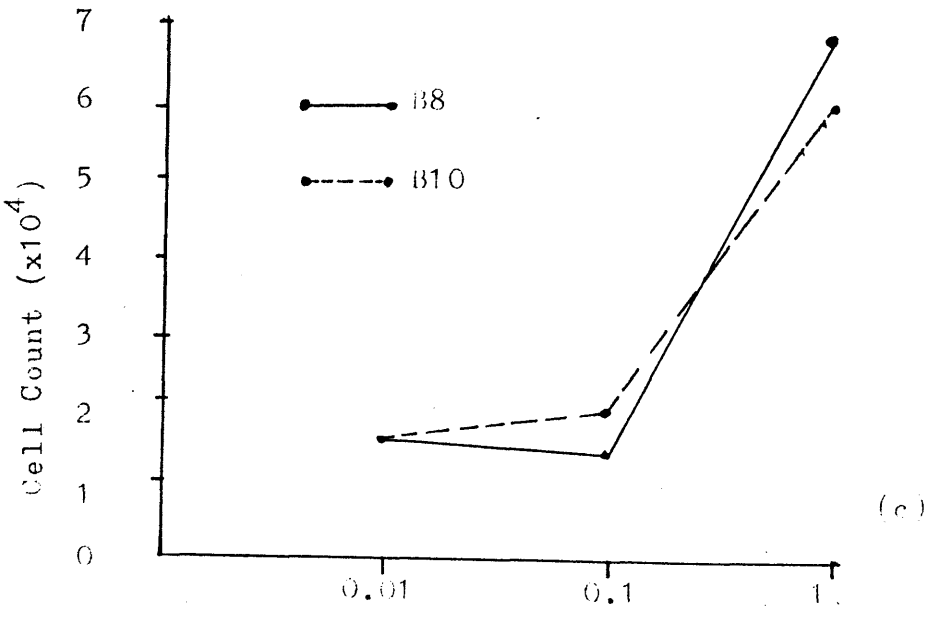
After testing the potential supplements outlined above, it was decided that to achieve cell growth, under serum-free conditions compatable with that achieved under serum-supplemented conditions, the basic formula of MCDB104 would have to be altered to suit the new growth conditions. Thus a special batch of MCDB104 medium without calcium


2.0
1.5
1.0
0.5
0
Cell Count (x10^5)
B8
B10
0.01
0.1
1
x Normal Concentration.

(a)


2.0
1.5
1.0
0.5
0
Cell Count (x10^5)
B8
B10
0.01
0.1
1
x Normal Concentration.

(b)


7
6
5
4
3
2
1
0
Cell Count (x10^4)
B8
B10
0.01
0.1
1
x Normal Concentration.

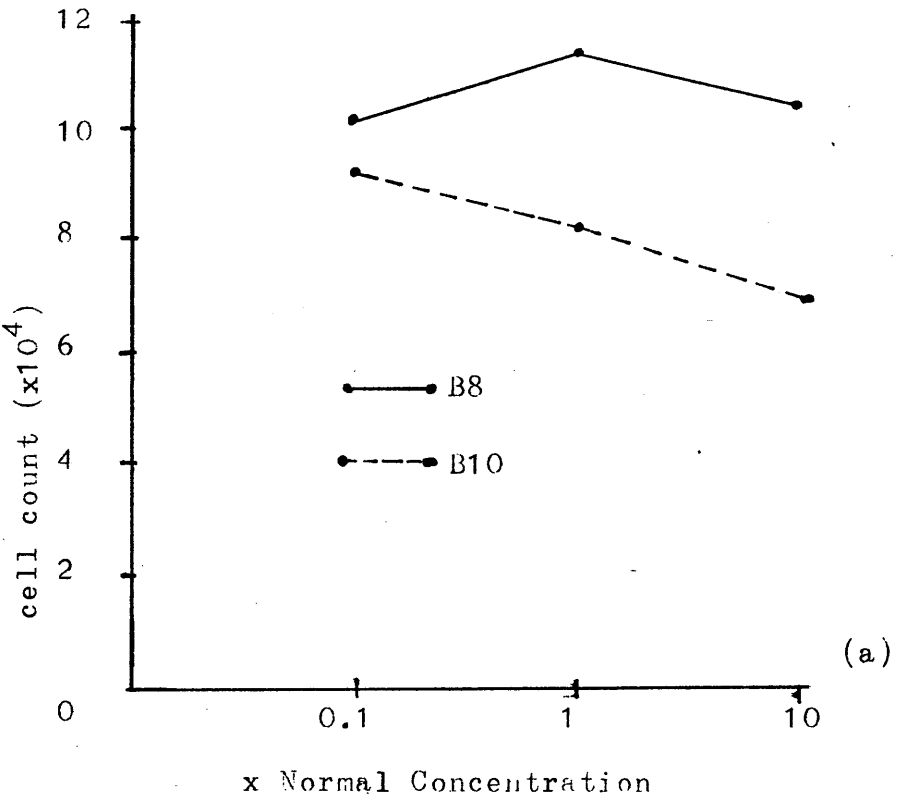
(c)

figure 18. The effect of (a) CaCl, (b) Na_2HPO_4 and (c) $MgSO_4$ on B0008 and B0010 cells under serum-free conditions.

chloride, magnesium sulphate, disodium hydrogen phospate potassium chloride, L-proline, L-tyrosine, L or D-valine, L-glutamine, sodium pyruvate, folinic acid, niacinamide, thymidine, adenine, choline chloride and inositol was prepared for us, and each omitted constituent was tested by making up the complete medium minus the test substance, then adding it to the medium at a variety of concentrations. Calcium chloride, magnesium sulphate, disodium hydrogen phosphate, L-glutamine and sodium pyruvate were tested at 1X, 0.1X and 0.01X the normal concentration while potassium chloride, L-proline, L-tyrosine, L-valine, D-valine, folinic acid, niacinamide, thymine, adenine, inositol and choline chloride were tested at 10X, 1X and 0.1X the normal concentration. The normal concentration for each test substance is shown in table 12. The results obtained for each test item are shown in figures 18 - 24. Standard supplements were added at the previously determined optimum concentrations (table 11).

4:17 Comparison of Melanoma Cell Growth Under Serum-Free Conditions in MCDB104 Medium, Modified MCDB104 Medium and in Foetal Calf Serum Supplemented Medium.

The modified MCDB104 medium was made up with the test components at the optimum concentration for each cell line- table 13. Supplements, bovine serum albumin, human transferrin, Intralipid, insulin and B-mercaptoethanol were added at the previously determined optimum concentrations for MCDB104 - each supplement was re-tested in the modified medium and the optimum concentrations were determined and found to be the same as those using the



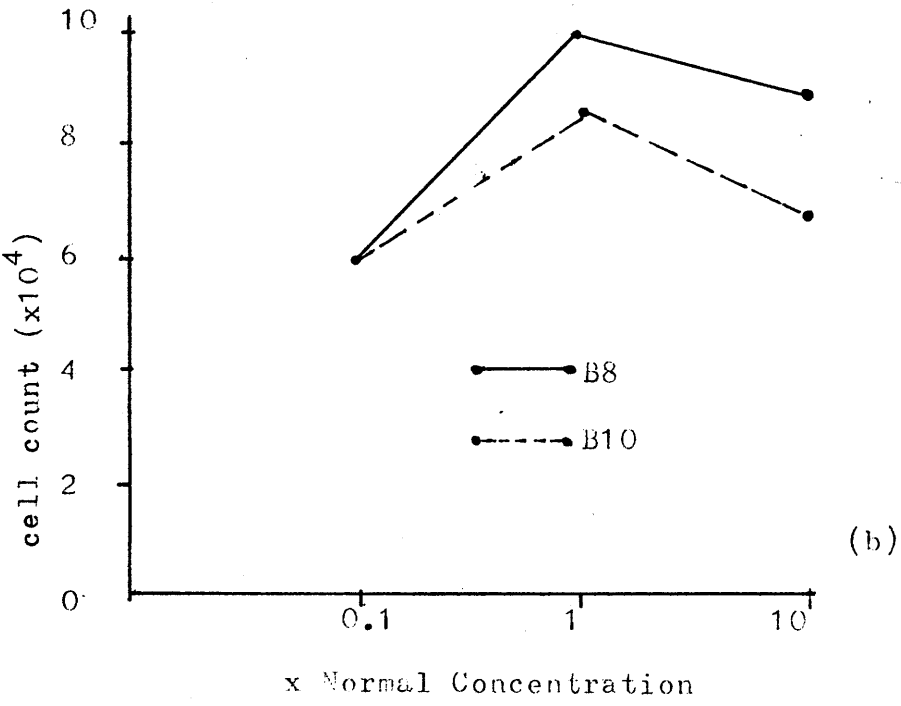


figure 20. The effect of (a) l-proline and (b) l-tyrosine on [illegible] cells under serum-free conditions.

Table 12: Normal Concentrations of the Omitted Test Items in Medium MCDB104

Item	Standard Concentration(mg/ml)	Test Range(mg/ml)
Calcium Chloride	0.147	0.00147-0.147
Magnesium Sulphate	0.246	0.00246-0.246
Disodium Hydrogen Phosphate	0.426	0.00426-0.426
Potassium Chloride	0.223	0.0223-2.230
Sodium Pyruvate	0.110	0.0011-0.11
Choline Chloride	0.013	0.0013-0.13
Folinic Acid	0.0000006	0.00000006-0.000006
Niacinamide	0.006	0.0006-0.06
Thymidine	0.000072	0.0000072-0.00072
Adenine	0.0013	0.00013-0.013
Inositol	0.018	0.0018-0.18
L-glutamine	0.365	0.00365-0.365
L-proline	0.034	0.0034-0.34
L-tyrosine	0.0054	0.0054-0.054
L or D-valine	0.011	0.0011-0.11

standard MCDB104 medium (table 11). MCDB104 unmodified had the standard supplements added at the optimum concentrations and MCDB104 medium supplemented with 10% foetal calf serum was set up as a control. The results are shown in table 14.

4:18 Further Improvement of Cell Growth in Modified MCDB104 Medium

As can be seen from table 14 our modified MCDB104 medium has increased cell growth under serum-free conditions to a level approaching that achieved using serum-supplemented medium. However it was felt that

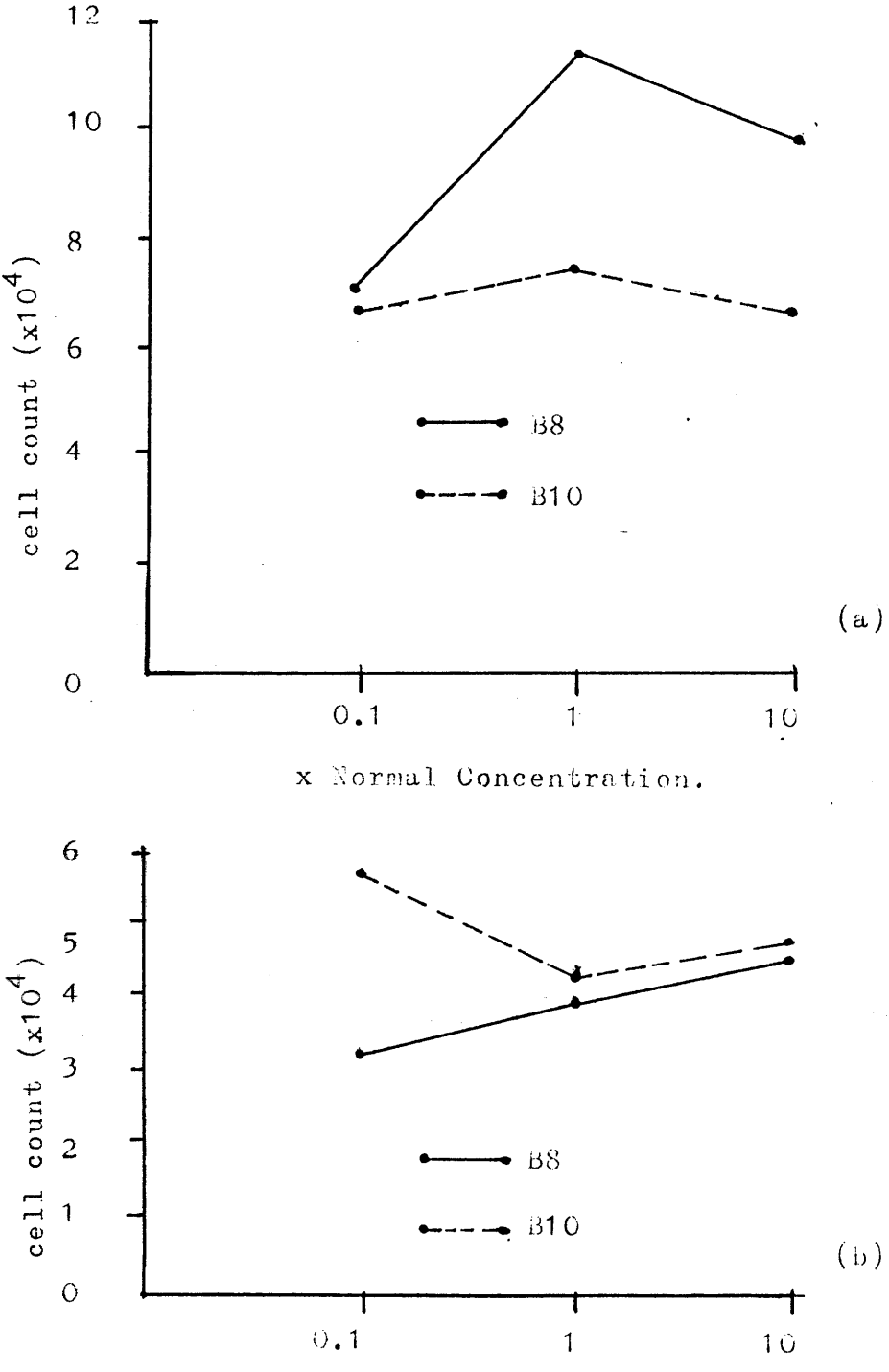
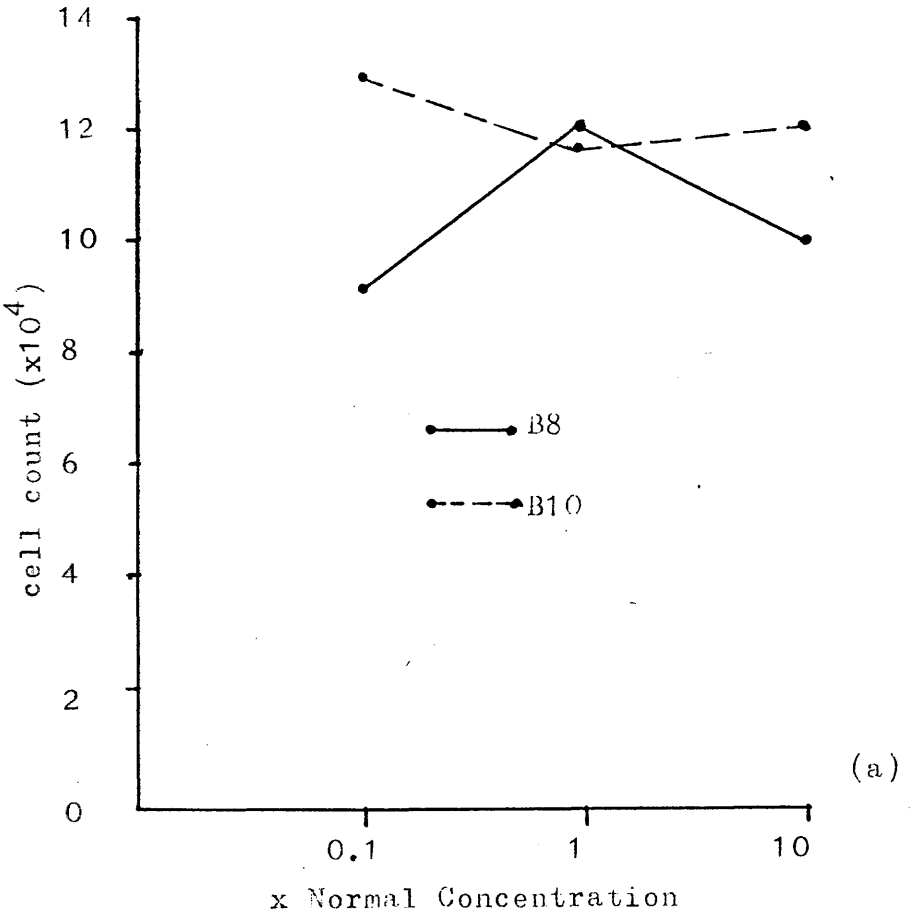
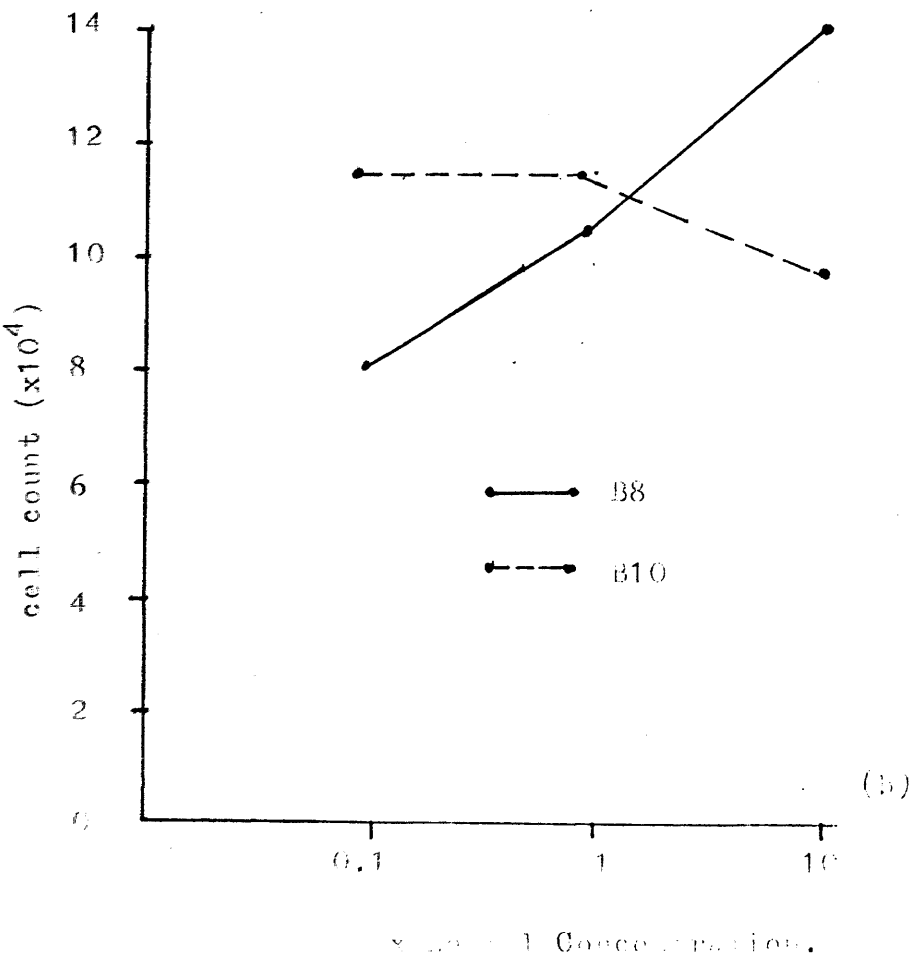


figure 21. The effect of (a) L-valine and (b) D-valine on B0008 and B00010 cells under serum-free conditions.


14
12
10
8
6
4
2
0
cell count (x10^4)
B8
B10
0.1
1
10
x Normal Concentration

(a)


14
12
10
8
6
4
2
cell count (x10^4)
B8
B10
0.1
1

(b)

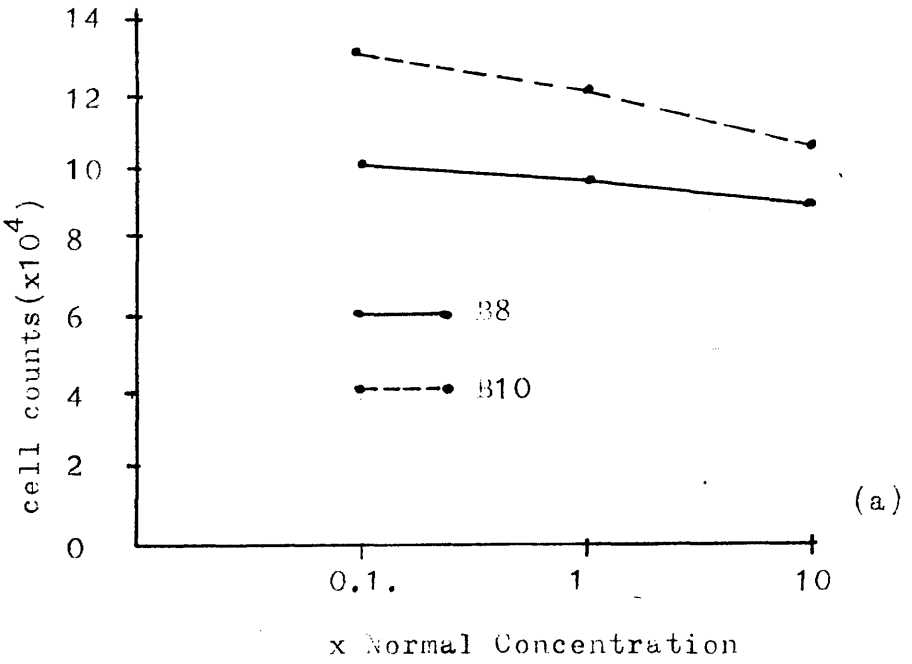
figure 22. The effect of (a) choline chloride and (b) potassium chloride on B0008 and B00010 cells under serum-free conditions.

this could be improved upon. Thus some of the previously tested supplements were re-tested. Ferrous ammonium sulphate, L-cystine with and without B-mercaptoethanol and potassium ferricyanide were re-tested. Over a wide

Table 13: Optimum Concentrations of the Tested Components of MCDB104 Medium for Two Human Melanoma Cell Lines

	Concentration (mg/ml)	
Component	B0008	B00010
Calcium Chloride	0.147	0.147
Magnessium Sulphate	0.246	0.246
Disodium Hydrogen Phosphate	0.426	0.426
Potassium Chloride	2.230	0.0233
L-proline	0.034	0.034
L-tyrosine	0.0054	0.0054
L-valine	0.011	0.011
L-glutamine	0.365	0.00365
Sodium Pyruvate	0.110	0.110
Folinic Acid	0.00000006	0.00000006
Niacinamide	0.06	0.06
Thymidine	0.00072	0.00072
Adenine	0.00013	0.00013
Choline Chloride	0.013	0.0013
Inositol	0.18	0.0018

concentration range none of these substances significantly improved cell growth (results not shown). However, calmodulin produced a similar dose response curve to that shown in figure 14. However, rather than continue assessing supplements for possible growth promoting effects,



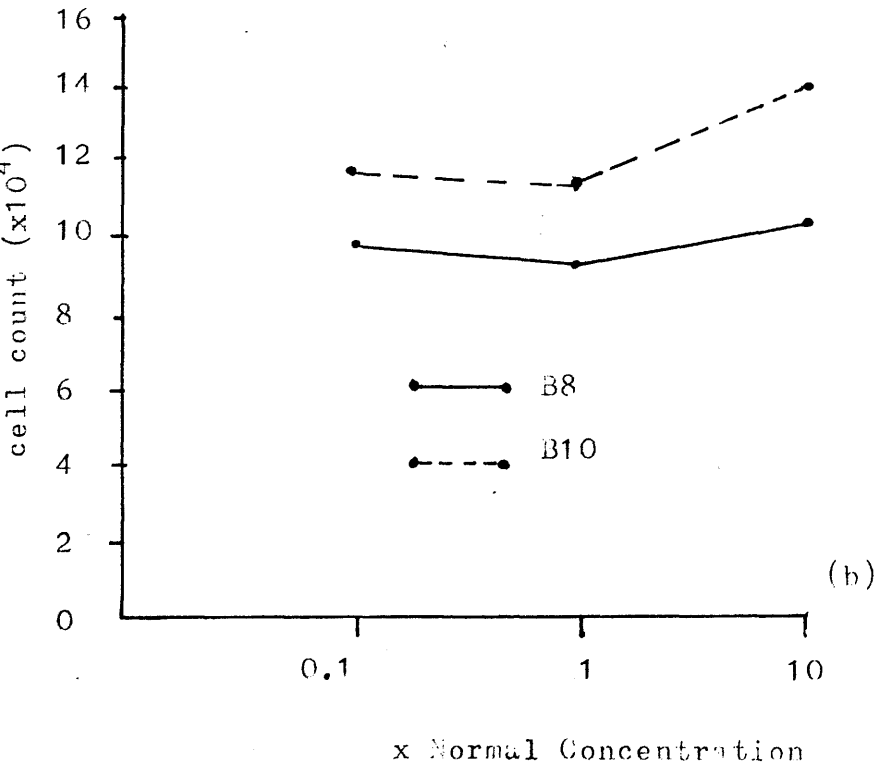


figure 23. The effect of (a) folinic acid and (b) thymidine on BOOCS and BOCO10 cells under serum-free conditions.

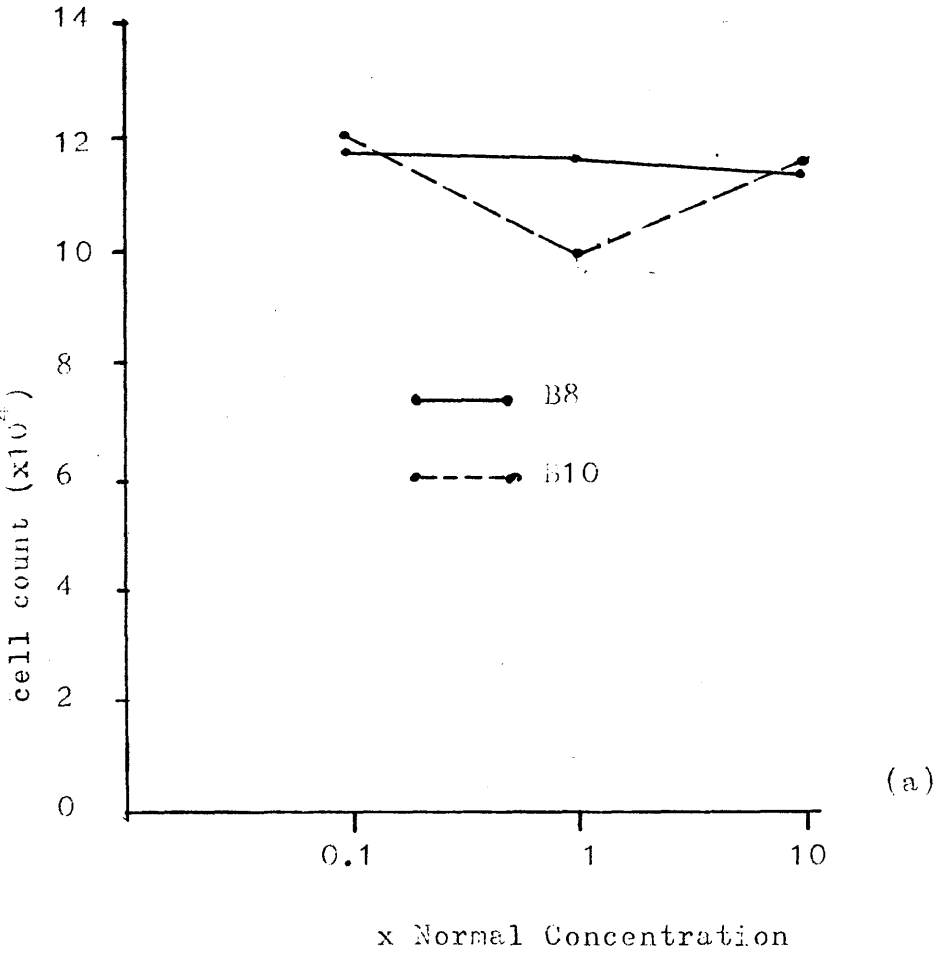
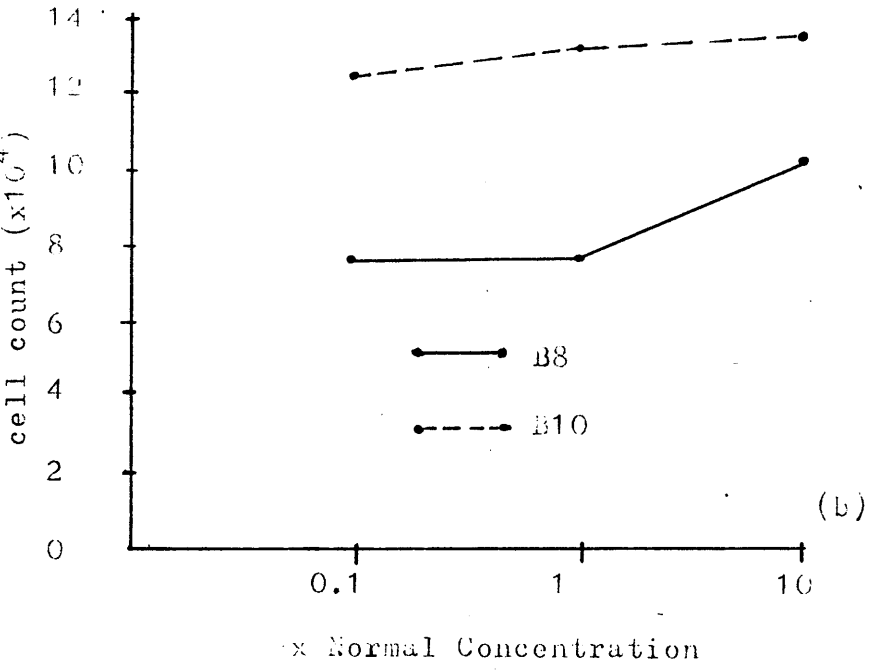
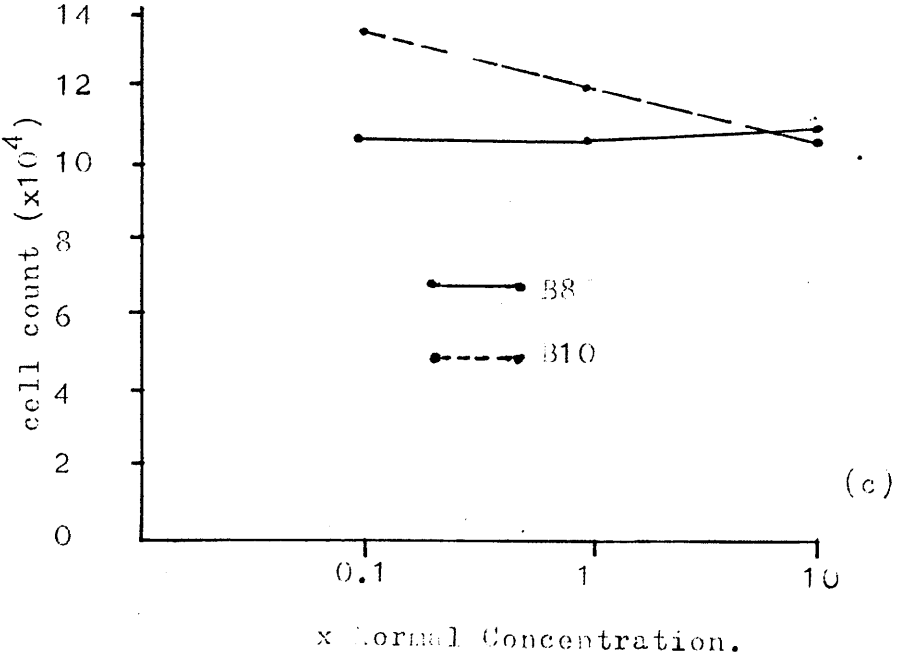


figure 24. The effect of (a) adenine, (b) niacinamide and (c) inositol on B0008 and B0001C cells under serum-free conditions.


14
12
10
8
6
4
2
0
cell count (x10^4)
B8
B10
(b)
0.1
1
10
x Normal Concentration


14
12
10
8
6
4
2
0
cell count (x10^4)
B8
B10
(c)
0.1
1
10
x Normal Concentration.

it was decided that the level of cell growth achieved using the modified MCDB104 medium was sufficient for our needs and that any future attempts to improve upon the level of cell growth attained would involve the optimisation of the entire components of MCDB104 medium, thus leading to the formulation of a "new medium".

Table 14: Comparison of Melanoma Cell Growth Under Serum-Free Conditions in MCDB104 Medium, Modified MCDB104 Medium and in Foetal Calf Serum Supplemented Medium.

	Cell Count($x10^5$)	
Medium	B0008	B00010
MCDB104	3.784	2.941
Mod.MCDB104	5.528	4.742
MCDB104 with 10% FCS	7.654	6.173

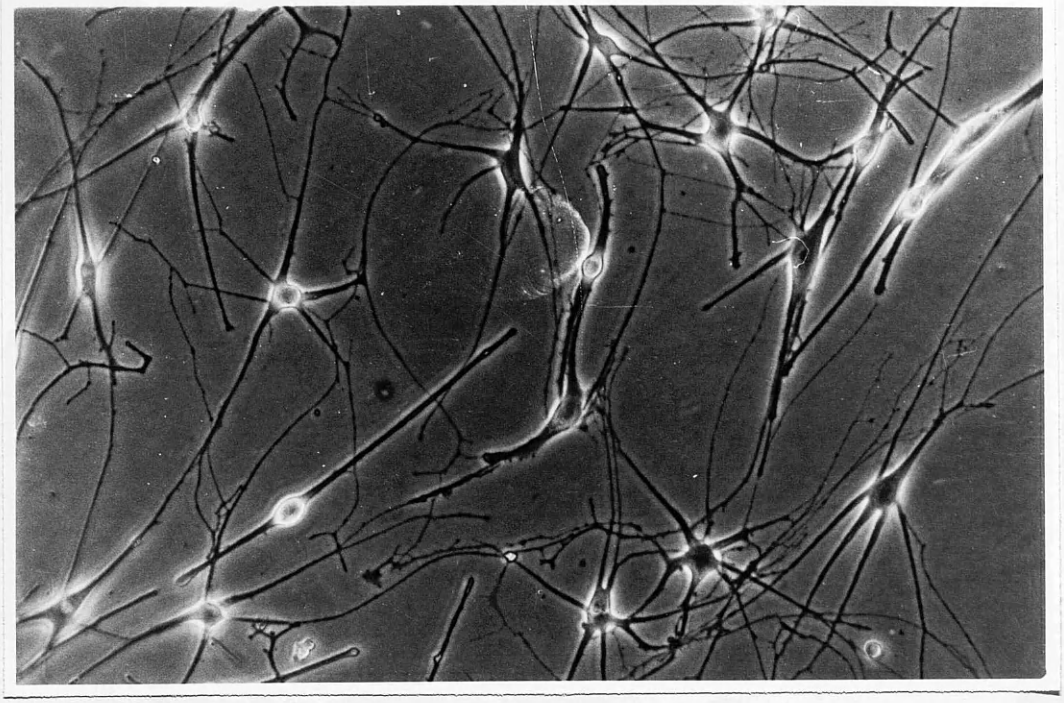
5. Results - Normal Human Melanocytes.

5:1 Choice of Culture Method.

Two methods were fully investigated for the culture of normal human melanocytes. These have been previously described (chapter 3). The Eisinger method was selected because this was the most successful method we tried, and also that of Wilkins because it did not involve the use of any tumour promoters of carcinogens. Using these two methods we could culture normal melanocytes routinely for periods of 3 - 6 months. However, fibroblast overgrowth was still a problem especially in cultures derived using the Eisinger method. Thus we further investigated reported methods for the control of this problem and also looked for ways of further increasing melanocyte proliferation.

5:2 Substitution of L-valine by D-valine in the Growth Medium.

A medium containing D-valine instead of L-valine is routinely available. Some reports have suggested that this D-amino acid is of use in the inhibition of fibroblast proliferation in epithelial mixed cell cultures. Thus we tried culturing normal melanocytes in this medium for 24 hours and then slowly increasing the exposure time to 96 hours. When cultures so treated were compared to melanocytes cultured in medium containing L-valine, there were no obvious differences in the numbers of fibroblasts or melanocytes present. (table 15).



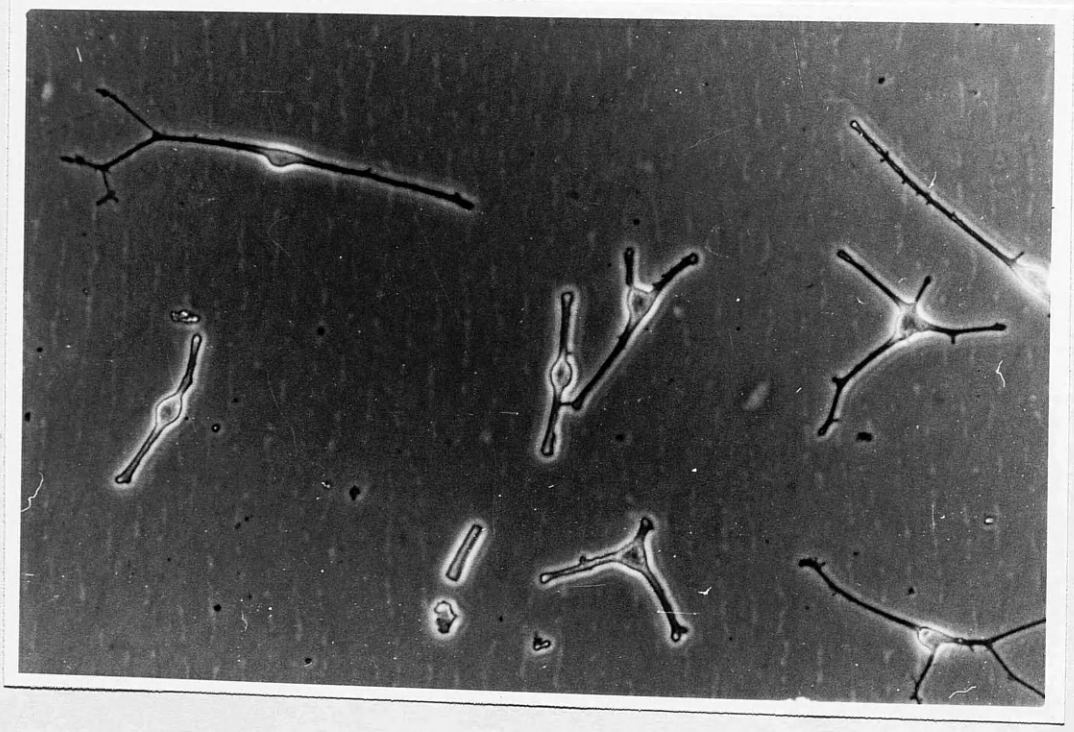


Table 15: Melanocyte and Fibroblast cell numbers from cultures grown in routine MEM or MEM D-valine

Cell type	Cell Count ($x10^4$) Routine MEM	MEM D-valine
Melanocytes	2.48 (69.66%)	2.08 (59.94%)
Fibroblasts	1.08 (30.34%)	1.39 (40.06%)

( ) percentage of total cell numbers.

5:3 Use of Coated Tissue Culture Dishes.

Specially coated dishes are commercially available which have been specifically developed for the culture of primary cell lines. These partially inhibit fibroblast attachment and proliferation. During this study these dishes were routinely used in the cultivation of normal human melanocytes. The manufacturers claim that these dishes successfully inhibit fibroblast attachment and proliferation by approximately 50% however this claim was not investigated by us.

5:4 Use of Antifibroblast Antibody.

We treated mixed cell cultures (mainly melanocytes and fibroblasts) with an antifibroblast antibody, kindly provided by Dr. Paul Edwards. The antibody was diluted 1:1000 and added to cultures for 90 minutes at $4^{o}C$. Cultures were then treated with a 1:40 dilution of rabbit serum for 3 hours at $37^{o}C$. After this treatment no cells - fibroblasts nor melanocytes - survived. Plate 1 shows before and after treatment photographs of mixed cell cultures containing normal human melanocytes. Dilutions of 1:10,000 and 1:100,000 of the antibody and 1:50 and 1:60 of the rabbit serum were also tested. At the higher dilution no cells survived while at the lower dilution the cell growth

of all the cell types present was unaffected. The rabbit serum alone was tested to ensure that it was not cytotoxic to normal melanocytes and no adverse affects were noted.

5:5 Use of Genticin and 5-Fluorouracil as a Method of Fibroblast Control.

Both of these compounds have been reported to be useful in the control of fibroblast contamination in mixed cell cultures (Halaban and Alfano, 1984 and Tsuji and Karasek, 1983). Genticin was added to mixed cell cultures at a concentration of 100ug/ml and no difference in the numbers of fibroblasts or melanocytes was noted (table 16). Increasing the concentration to 1000ug/ml made no difference. 5-fluorouracil was added to cultures at a concentration of 1.92×10^{-5}M. This proved toxic to both fibroblasts and melanocytes. Reducing the concentration produced the same effect.

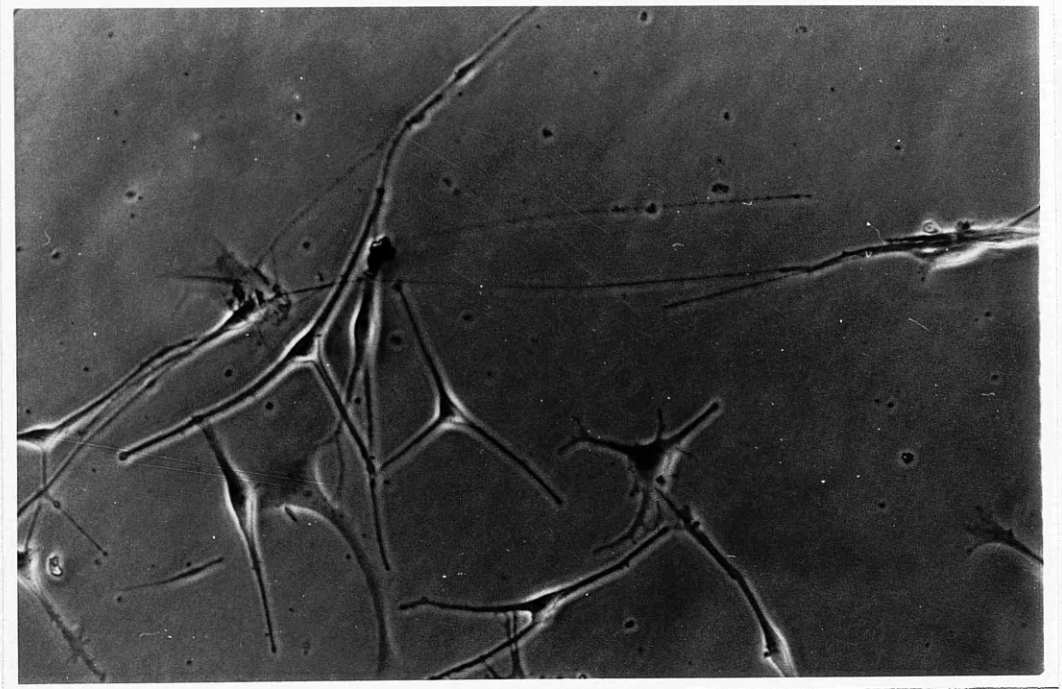
Table 16: Affect of 100ug/ml Genticin on Melanocyte and Fibroblast Cell Numbers.

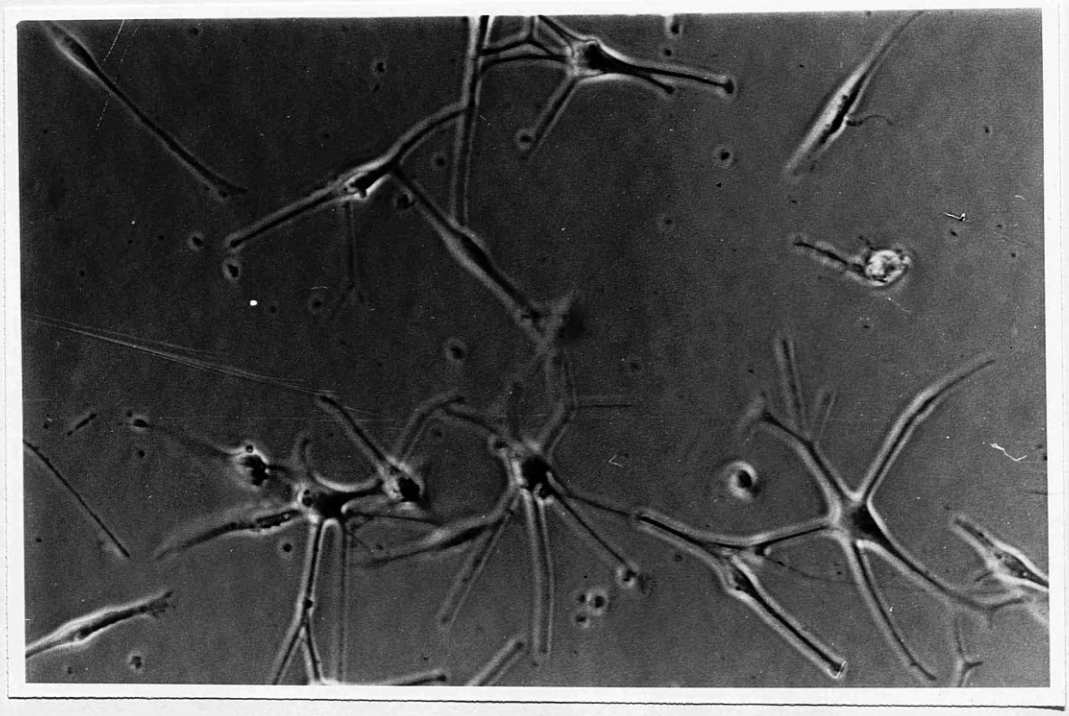
	Cell Count ($x10^4$)	
Cell Type	+ Genticin	- Genticin
Melanocytes	1.96 (57 .82%)	2.04 (61.19%)
Fibroblasts	1.43 (42.18%)	1.25 (38.05%)

( )percentage of total cell numbers.

5:6 Use of Conditioned Medium to Stimulate Normal Melanocytes.

Medium from the two melanoma cell lines B0008 and B00010 was collected, filter sterilized and added to normal melanocyte cultures in various proportions. If the melanoma cells were cultured in RPMI 1640 medium, the conditioned medium was toxic to the normal melanocytes.





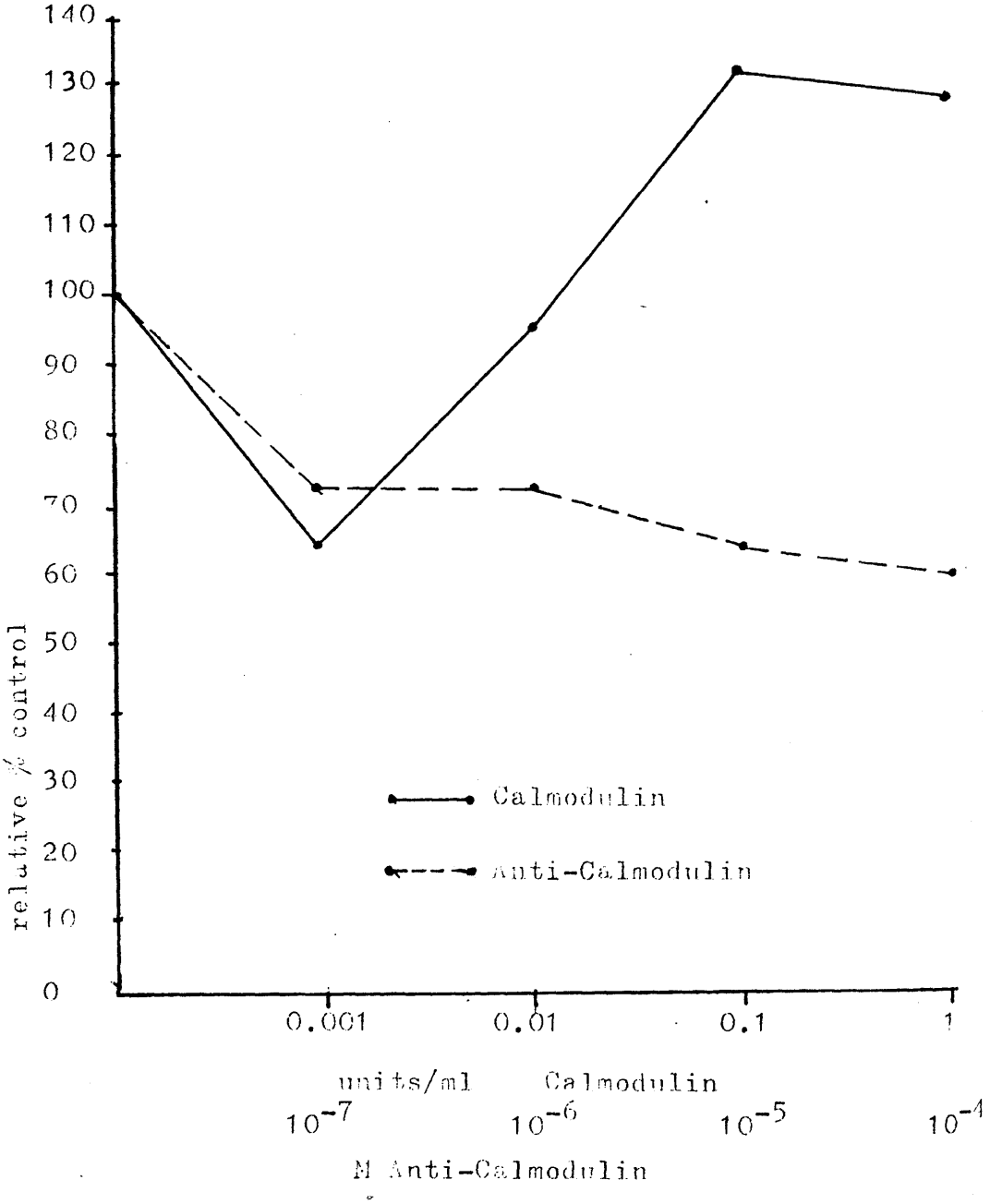


figure 25. Effect of calmodulin and anti-calmodulin on normal human melanocytes.

free conditions. Melanocyte proliferation was not achieved using any of these media. The addition of cholera toxin (10^{-8}M) and phorbol ester (10ng/ml) did not bring about any increase in melanocyte numbers - in fact this combination, under serum-free conditions, was toxic to melanocytes in culture. We also found that the forementioned supplements could not replace the limited use of foetal calf serum in the Wilkins method of melanocyte culture.

6. Discussion

This report chronicles the development of a serum-free medium which allowed us to culture the two human melanoma cell lines B0008 and B00010 in the absence of serum for 1 - 2 weeks. The final composition of this medium was a modified MCDB104 medium supplemented with bovine serum albumin, human transferrin, Intralipid, insulin and B-mercaptoethanol. These are the basic supplements.

The major protein in the medium is the plasma protein bovine serum albumin. As serum albumin is not taken up by cells it must function externally where it is thought to be important in the scavenging of the toxic by-products of cell metabolism (Darfler and Insel,1983). The main function of transferrin is in the transportation of iron into the cells. However, it has been shown, in the B16 mouse melanoma cell line, that growth stimulation by transferrin occurs even if very low levels of iron are present in the medium. This suggests that although iron transportation is the major function of this protein, its stimulatory effect may also be due to other, as yet undetermined, functions (Mather and Sato, 1979). It has also been suggested that transferrin may act externally by binding toxic metal ions in the medium (Iscove and Melchers, 1978).

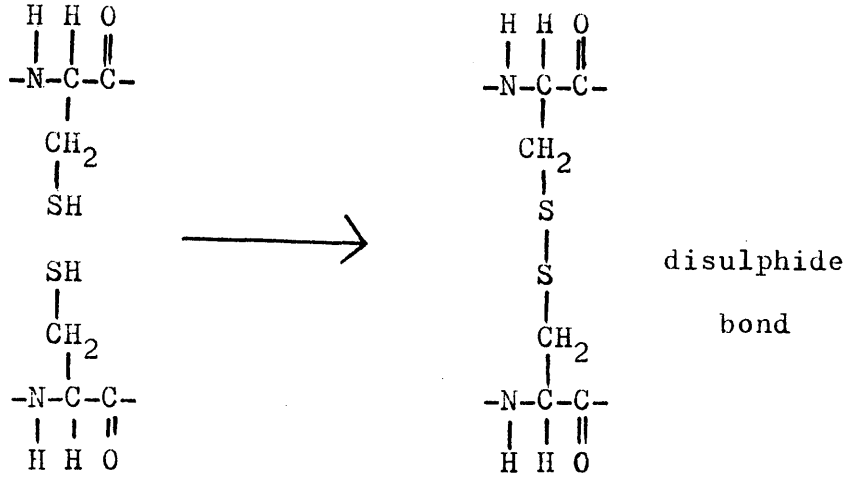
The original lipid supplement used was soybean lipid (Iscove and Melchers, 1978) which contains soybean lecithin and cholesterol. However there were toxicity problems with this supplement. While initial results

(figure 3) were promising, each new batch had to be standardised against the previous batch. Some batches had to be rejected on toxity grounds. Because of these drawbacks, the lipid supplement was changed to Intralipid. Although, for patent reasons, the makers would not reveal the exact composition of this supplement, we were told that the major fatty acids present were palmitic, stearic, oleic, linoleic and linolenic acids. Both linoleic and linolenic acids are not manufactured by the cell and thus are classed as essential fatty acids. Palmitic and oleic acids are important in cell metabolism. The ionised form of oleic acid, oleate, is used to form a variety of unsaturated fatty acids by a combination of elongation and unsaturation reactions. Palmitate, the ionised form of palmitic acid, is oxidised to yield ATP.

Although melanoma cells are not regarded as classical insulin target tissues it is an interesting fact that their method of response to insulin is thought to be in the classical fashion (Mather and Sato,1979). Insulin is not stable at 37°C thus its effects on cells, in culture, must occur shortly after its addition to the medium. Hayashi (Hayashi et al, 1978) has shown, in the rat pituitary cell line GH_3, that the presence of insulin in the growth medium increases glucose incorporation into both glycogen and fatty acids. This has been confirmed for cell line M2R derived from the B16 mouse melanoma (Mather and Sato,1979). Thus it is probable that insulin may have the same function in our serum-free medium. We made no attempt to look at

the effect of insulin on either glycogen or fatty acids in this system.

The reducing agent B-mercaptoethanol is also routinely added to our medium. It is thought to function by preventing the formation of disulphide bonds such as those which occur in the conversion of L-cysteine to L-cystine



L-cysteine L-cystine

Our interest in the culture of normal human melanocytes arose because of the need for a normal control cell population for studies on human malignant melanoma cells. Of the methods available for melanocyte cell culture we elected to use that described by Eisinger (Eisinger and Marko, 1982) and that of Wilkins (Gilchrest et al, 1984, Wilkins et al, 1985). Although we are able to culture melanocytes routinely using the method of Eisinger, the use of the tumour promotor phorbol ester in the medium raises the problem of the "normality" of the melanocytes as it has been reported that in cell culture systems phorbol ester induces several phenotypic changes which resemble those seen in cells transformed by viral or chemical carcinogens

(Weinstein et al, 1977). The method of Wilkins has the advantage in that no tumour promotors or carcinogens are added to the medium, however the melanocyte yield achieved using this method is much lower than that achieved using the method of Eisinger.

Cholera toxin is added to both media and is believed to act by increasing the intracellular levels of cyclic AMP (cAMP). It does this by stimulating the enzyme adenylate cyclase which is active in the following reaction :-

$$ATP \xrightarrow{Mg^{2+}} cAMP + PPi + H^{+}$$

It is also thought that the rise in cAMP is one of the signals for the initiation of melanocyte cell division (Eisinger and Marko, 1982).

In the method described by Eisinger the other addition to the medium is the phorbol ester, phorbol 12-myristate 13-acetate. How this compound acts in the medium has not been well researched but there are some indications. Recent reports have indicated that phorbol esters stimulate phosporylation events. Grunberger (Grunberger et al,1984) has report that in U-937 monocytes phorbol ester stimulates tyrosine phosphorylation by increasing tyrosine kinase activity. This work suggests that stimulation of membrane associated kinases especially the phospholipid dependent protein kinase C, initiates the action of bioactive phorbol esters. It is known that cAMP also activates the protein kinases and in fact many of the effects elicited by cAMP results from the activation of protein kinases (Stryer). The function of the protein kinase is to modulate the

activity of different proteins by phosphorylating them. If phorbol ester acts in a similar way in melanocytes then stimulation of the protein kinases may be part of the method by which melanocyte cell division is initiated.

Work by Lee and Weinstein (1975) has associated the mode of action of phorbol esters with that of epidermal growth factor which is used in the Wilkins method. They have shown that phorbol esters inhibit the binding of epidermal growth factor to its cellular receptors. It has been suggested that inhibition may be due to a decrease in the number of available epidermal growth factor receptors rather than to a change in receptor affinity. Thus some of the effects of phorbol ester may result from alterations in the function of cell surface receptors involved in growth regulation. In addition work by Sahai (Sahai et al, 1982) has shown that phorbol ester and epidermal growth factor act synergistically in the inhibition of A431 human epidermoid carcinoma cells. This synergistic effect may be of use in improving the yield of normal melanocytes if it also occurs in this system.

In the method of Wilkins perhaps the most important addition is the melanocyte growth factor obtained from bovine brain. This factor has been partially characterized (Wilkins et al, 1985). It has been shown to be a heat and alkaline labile mitogen which is capable of stimulating the growth of human melanocytes at low seeding densities. It is non-dialyzable, partially stable in acid below pH3.0 and has an apparent molecular weight of 30,000 as determined by gel exclusion chromatography.

Work has also shown that purified melanocyte stimulating hormone (MSH) cannot substitute for the activity of the melanocyte growth factor which implies that the factor is not an analog of MSH (Wilkins et al, 1982).

The functions of the other components in the Wilkins melanocyte medium - insulin, transferrin, triiodothyronine, hydrocortisone and nerve growth factor - are not well documented. Nerve growth factor has been shown to be a survival factor for the neural crest derived cells (Chun and Patterson, 1977). Melanocytes are derived from the neural crest. The role of both transferrin and insulin in culture medium have been described earlier while it is thought that hydrocortisone may act by increasing metabolic processes such as protein synthesis but, perhaps more importantly for the culture of melanocytes, it has been reported that hydrocortisone present at a low concentration may have an inhibitory effect on the growth of fibroblasts (Gower). Triiodothyronine is related to the hormone thyroxine (tetraiodothyronine) and may act by increasing the cellular uptake of glucose.

The selection of additives in the growth medium minimize the proliferation of fibroblasts in the culture system. However, perhaps the most important control method for the contaminating cell types is the removal of serum from the culture system. The main advantage of this seems to be in the removal of fibronectin from the cell environment. The lack of fibronectin accounts in part for the lack of keratinocyte contamination (Gilchrest et al, 1980, Maciag et al, 1981). The other contributary factor in the control

of keratinocyte contamination is the dialysis of the bovine brain extract to remove any keratinocyte growth factors present. Normal melanocytes are not adversely affected by the removal of serum as it has been reported that they do not recognize fibronectin as a cell attachment factor (Wilkins et al, 1985).

Once adequate cell growth in the basic serum-free medium was achieved we improved this by testing various supplements in Ham's F-10 supplemented with the basic supplements. The first additives to be looked at were the amino acids. Of those tested L-methionine, L-threonine, L-tryptophan and L-valine increased cell growth under serum-free conditions. These are all essential amino acids and as such are a basic requirement for cell growth.

The next set of supplements to be tested were various metal ions. The first to be looked at was selenium in the form of sodium selenite. This metal is believed to partially protect against the death of cells at clonal density perhaps by scavenging hydrogen peroxide which is a toxic by-product of metabolism. This has been shown to be the case in lymphoid cells (Darfler and Insel, 1983). However even at the lowest test concentration (10^{-10}M) this compound proved toxic to both melanoma cell lines.

Ferrous ammonium sulphate was also tested for growth promoting effects. Tested on its own, ferrous ammonium sulphate proved slightly stimulatory to both B0008 and B00010 cells. However, it is impossible to consider its growth promoting effects without taking into account the presence of transferrin. Reports have suggested that

supplementing the medium with ferrous ions and transferrin at the appropriate concentrations gives optimum cell growth (Iscove and Melchers, 1978, Brown et al, 1983). We tested transferrin at 1ug/ml and 10ug/ml with ferrous ammonium sulphate over a range of 10^{-8} - 10^{-11}M. Optimum cell growth occurred at 1ug/ml and 10^{-9}M respectively. However this disagrees with the findings of Mather (Mather and Sato, 1979). They found that supplementation with exogenous ions unnecessary. This work suggests that the function of transferrin in stimulating cell proliferation in serum-free medium may be due to mechanisms other than iron transportation, as part of their work was to test the effect of transferrin in medium which had been depleted of iron. However it is possible that the hormones present in the Mather-Sato serum-free medium may compensate for the lack of iron in some way. In later work carried out using MCDB 104 medium we found that adding exogenous iron to the system did not result in increased cell growth. This would suggest that sufficient iron is present in this medium to stimulate optimum cell growth in this system.

Potassium ferricyanide was found to be slightly stimulatory to both cell lines at a concentration of 10^{-10}M. How this compound acts in the culture system to bring about cell stimulation is unknown. A trace element mixture developed in the laboratory of Ham (McKeehan and Ham, 1976) containing manganese, molybdenum, nickel, selenium, tin and vanadium proved stimulatory over a range of concentrations (figure 10). The interesting point to note is that trace element mixture is stimulatory despite containing selenium

which is inhibitory on its own. It may be that its toxic effects are neutralized in the mixture or that in this form it does in fact contribute to cell stimulation.

After looking at the metal ions as potential growth stimulators we looked at the polyamine putrescine. This substance was found to be slightly stimulatory to both cell lines and has been reported to be useful in the culture of normal melanocytes (Nielsen and Don, 1984). It is thought to have an effect on cellular osmosis. How this affects growth stimulation is not clear.

Other studies in our laboratory centred around the use of the stem cell assay technique (Hamburger and Salmon, 1977) as a tool to look at the response of freshly biopsied melanoma tissue to the various drugs used in chemotherapy. In this technique either agar or methylcellulose (methocel) is used as a top layer to hold a single cell population in suspension thus resulting in clones occuring, or not, depending on the effect of the drugs present. Thus we looked at the effect of adding methocel to the serum-free medium at a concentration of 0.6%. We found that this greatly increased cell growth. The increase was probably due to the fact that the presence of the methocel stopped cell clumping from occurring thus allowing nutrients to be more available. Also cells took longer to settle onto the culture surface thus the nutrients held in the medium are active on all cell surfaces for a longer period of time. However the use of thus substance was not without its problems. While at 37°C, methocel remains in a semi-solid state, however once removed from the incubator it

becomes runny thus making microscopic examination difficult. Also methocel is a relatively impure substance and unless cell cultures are thoroughly washed before harvesting debris present in the suspension can lead to falsely high cell counts. These associated problems made the use of methocel impractical and thus despite the increased cell growth its use was discontinued.

While testing the various substances we found that the effect of the amino acid L-cystine in the medium could be modified by the presence of B-mercaptoethanol. It may be that the presence of the reducing agent breaks the disulphide bonds present in L-cystine converting it to L-cysteine. The increased level of L-cysteine may play a part in the inactivation of free insulin present in the medium (Mather and Sato,1979). When L-cystine is added to the medium in the absence of B-mercaptoethanol cell growth is also stimulated. This may be brought about by the amino acid on insulin. It has been reported that the inclusion of L-cystine in the medium may reduce the concentration of insulin required to have a stimulatory effect on cells (Mather and Sato, 1979). This work was carried out on B16 mouse melanoma cells, thus it may be relavent to human melanoma work.

The main problem in the culture of normal human melanocytes is overgrowth by the contaminating cell types- mainly fibroblasts. In the method of Eisinger keratinocyte contamination is controlled by the addition of phorbol ester. It is thought to inhibit keratinocyte attachment by altering the calcium permeability of the cells.

Fibroblast contamination is controlled by a differential trypsinization technique. This technique is a practiced art and must be continually monitored. The principle of the technique is that melanocytes detach from the culture surface before the fibroblasts and any remaining keratinocytes. The process must be terminated immediately the melanocytes begin to detach from the culture surface. However it is easy to terminate the process too soon resulting in a low cell yield or alternatively allowing trypsinization to proceed for too long thereby passaging both melanocytes and fibroblasts. Thus cultures are never completely free of fibroblasts, the contamination is merely controlled. In the method of Wilkins, the contaminating cell types are controlled by the removal of serum from the medium and the dialysis of the bovine brain extract to remove any keratinocyte growth factor activity. If serum is routinely re-introduced, fibroblasts re-appear thus again contamination is controlled rather than erradicated.

The problem of fibroblast overgrowth arises because of the very long doubling time of melanocytes. This was illustrated by Jimbow (Jimbow et al, 1975) who looked at human melanocytes in vivo. After looking at sections of non-stimulated, unexposed skin from 18 subjects he found only two dividing melanocytes. Thus, altough the mechanics required for cell division are present they are not frequently used. Alternatively, in vivo, fibroblasts are replaced regularly and in vitro multiply readily thus becoming quickly established under tissue culture conditions.

Thus a method for the total erradication of fibroblasts from the culture system would be very useful. To this end we investigated various techniques.

Firstly we looked at the effect of substituting D-valine for L-valine in the culture medium. This medium MEM D-valine was developed by Gilbert and Migeon (1975). The reason for using this medium is that fibroblasts do not have the enzyme D-amino acid oxidase which is required to convert D-valine into the L-isomer. As L-valine is an essential amino acid, in theory fibroblasts should not be able to survive without it. When mixed cell cultures containing normal melanocytes were grown in MEM D-valine for periods of up to 96 hours, we found no real change in the number of fibroblasts or melanocytes present. Work carried out on malignant melanocyte cell lines showed that these cells can only survive in medium containing the D-amino acid at greatly reduced concentrations. This may be explained by the fact that the doubling time of normal melanocytes is in the region of 10 - 14 days, thus exposure to D-valine for 3 days and then replacement with L-valine is unlikely to have a lasting effect. The fact that the fibroblasts were not affected is perhaps surprising. This may be due to insufficient exposure to the D-isomer or some other factor in the culture system.

We next looked at the use of specially coated tissue culture dishes in our culture system which we found very useful. The dishes were commercially available and due to patents pending the composition of the coating material is unknown. However, literature distributed by the supplier

indicates that the coating has similar properties to coating with collagen types IV and I, polylysine, extracellular matrix and the normal tissue culture surface. Exactly how the coating affects fibroblast attachment and proliferation is not known. No quantitative work was carried out by us to determine the reduction in fibroblast numbers achieved by the routine use of these dishes.

An antifibroblast antibody (Dr. P.Edwards kindly provided it) was tested and we found that it was not an effective means of removing contaminating fibroblasts from our normal melanocyte cultures. The antibody was tested and used at the concentration recommended in the original paper (Edwards et al, 1980) and also at much lower concentrations. Even at the lowest test concentration ($1:10^6$) the antibody was still cytotoxic to the normal melanocytes. The antibody was described in the original paper as not being truely fibroblast specific, thus it may be that melanocytes are similar enough to fibroblasts to be attacked by the antibody.

Finally we looked at the effects of cytotoxic drugs on fibroblast proliferation. In the method described by Tsuji and Karasek (1983) melanocyte cultures are freed of keratinocyte and fibroblast contamination by a three week treatment with the drug 5-fluorouracil. However we found this method to be ineffective. The action of the drug is dependent on the doubling time of the cells. Keratinocytes and fibroblasts, with relatively short doubling times, should incorporate the drug within a few days whereas the melanocytes would require many days or weeks to be similarly

affected.

We also looked at a report which suggested that fibroblast contamination could be controlled by treatment with the antibiotic genticin (G418 sulphate) (Halaban and Alfano, 1984). Its mode of action is thought to be similar to that outlined above for 5-fluorouracil. Again we found that it had no detectable effect on fibroblast proliferation in our culture system.

As we were unsuccessful in finding a method of controlling fibroblast contamination we turned our attention to methods of increasing melanocyte proliferation. Firstly we looked at the effect of conditioned media on the culture system. We collected medium conditioned by the melanoma cell lines B0008 and B00010. These cells grow well in culture and have a relatively short doubling time compared to normal melanocytes. It is possible that these cells may produce "growth factors" which may be released into the culture medium. Such "factors" may be stimulatory to normal melanocytes. However, melanocyte proliferation was unaffected by melanoma conditioned medium although cell morphology was affected in that the melanocytes became more flattened. If, however, this conditioned medium had proved stimulatory then, once again, the normality of the melanocytes would have been questioned. As melanoma cells are transformed cells any "factors" produced by these cells which caused normal melanocyte stimulation may lead to alterations within the cells and initiate the first steps in cell transformation. A recent report by Eisinger (Eisinger et al, 1985) suggests that melanoma cells do indeed produce

factors which are stimulatory to normal melanocytes. However these factors are intracellular and the melanoma cells must be broken down for the factors to be released. This process has not, as yet, been investigated by us.

As stated earlier both methods used for the culture of normal melanocytes include cholea toxin which is believed to act by increasing intracellular cAMP levels this in turn is believed to signal the initiation of melanocyte cell division (Eisinger and Marko, 1982). Thus we looked at the effect of calmodulin which is also thought to increase intracellular cAMP levels (Walker et al, 1984). In conjunction with phorbol ester and cholera toxin, calmodulin increased melanocyte numbers. This increase may be due to the amplification of the signal to initiate cell division. However, if calmodulin replaced either phorbol ester or cholera toxin, melanocyte numbers decreased. This again suggests amplification as a mode of action. The anti-calmodulin agent, W7 was also tested in the melanocyte cultures with a view to its use in controlling the contaminating fibroblasts. However, all cell types were inhibited at the higher concentrations tested.

One of the initial aims of this study was to investigate the possibility of culturing normal melanocytes under serum-free conditions. At all stages in the development of the melanoma serum-free medium, normal melanocytes were also tested in it. At no stage was normal melanocyte cell growth achieved in our media. The addition of phorbol ester and cholera toxin to the serum-free system proved toxic to the melanocytes. However, if phorbol ester

and/or cholera toxin are added to melanoma cells cultured in serum containing medium the toxic effect was more gradual and in fact cells could survive under such conditions for 4 - 5 days before any adverse effects were seen (data not shown). Thus it is possible serum protects the melanocytes from some of the toxic properties of phorbol ester and cholera toxin. At present the nearest we have come to the culture of normal melanocytes under serum-free conditions is by the use of the medium described by Wilkins (Wilkins et al, 1985) however this is not really serum-free as serum is added at the initial seeding and at each subsequent passage.

Although a reasonable level of cell growth was achieved in Ham's F-10 without serum and supplemented with various additives there were, several problems with the system. The large number of supplements required made it difficult to maintain consistancy from batch to batch. The addition of so many supplements also increased the chances of introducing contaminants into the system. However, perhaps the most important problem was that it was virtually impossible to maintain the same concentration of amino acid from batch to batch. We had no method of determining amino acid degradation and thus we were unable to determine the concentration of amino acids required to be added to the medium to maintain optimum growth conditions. We decided that the level of serum-free cell growth achieve in this medium could be maintained or improved upon by using a different basic medium. Thus we decided to switch to the

later modification of Ham's F-10, MCDB104 (McKeehan et al, 1977). It has all the advantages of Ham's F-10 but also has increased concentrations of various amino acids and contains trace element mixture which has been shown to be stimulatory to cells under serum-free conditions.

We retested the basic supplements of bovine serum albumin, human transferrin, Intralipid, insulin and B-mercaptoethanol in medium MCDB104 and found that this produced a higher level of cell growth than was achieved in Ham's F-10 supplemented as previously outlined. Although cell growth was increased, it was still less than 50% of that achieved in serum containing medium. Thus we looked at the possibility of further supplementation. The first substances which we looked at were calmodulin and the anti-calmodulin agent W7. Work by MacNeil (MacNeil et al, 1984, Walker et al, 1984) has shown that W7 has an inhibitory effect on the B16 mouse melanoma cell line. We found this to be the case for both of the human melanoma cell lines used in this study. Under serum-free conditions we found calmodulin to be stimulatory to both cell lines. It has been reported that calmodulin like activity is present in foetal calf serum (Walker et al, MRS abstract). Thus part of the stimulatory effects may be due to the replacement of one of the functions of serum. There have also been reports that extracellular calmodulin can stimulate protein synthesis (MacNeil et al, 1984) and this may also act to stimulate cell growth.

In our work on the culture of normal melanocytes, phorbol ester and cholera toxin are used to stimulate

melanocyte division. Thus we investigated their growth promoting abilities in our serum-free medium. However, under serum-free conditions both phorbol ester and cholera toxin proved inhibitory to both cell lines at all tested concentrations. In serum-containing medium, both melanoma cells can survive in the presence of phorbol ester and cholera toxin for 4-5 days before any adverse effects are noted. Thus it would appear that serum protects the cells from the toxic effects of these compounds for a short period.

To achieve a level of cell growth approaching that obtained in serum-containing medium it was decided that the basic composition of the medium should be altered rather than to proceed with the testing of further supplements. Thus a batch of MCDB104 deficient in 15 items was prepared (table 12) and each item optimised. Of the items tested only four affected both cell lines in the same way. Niacinamide and thymidine increased cell growth when their concentration was increased by a factor of ten whereas with folinic acid and adenine stimulation was noted when the concentrations were decreased by a factor of ten.

Niacinamide is derived from the vitamin niacin and forms the important co-enzyme nicotinamide adenine dinucleotide (NAD) which is a major electron acceptor in the oxidation of fuel molecules. Thymidine is derived from the pyrimidine thymine and as such is important in the biosynthesis of nucleotides. Folinic acid is a member of the folate group and as such is involved in one carbon transfer. Adenine is one of the two major purines and,

like thymidine, it is involved in the biosynthesis of nucleotides. The fact that increasing the concentration of niacinamide and thymidine affects the proliferation of both cell lines suggests that the serum-free medium is deficient in these two components and it may be that this is a specificity for all human melanoma cell lines. In a similar manner the decreased concentrations of folinic acid and adenine may also be specific.

Inositol also affects the proliferation of both cell lines. For B0008 cells the optimum concentration is 10 times that found in standard MCDB104 medium while in B00010 the optimum concentration is a ten fold decrease from the normal. Inositol is an alcohol which is important in biological membranes. It is one of the common alcohol moieties of phosphoglycerides. This difference in the optimum concentration of inositol suggests that it is specific for each melanoma cell line. This may be due to different membrane compositions of the two cell lines under study.

Of the remaining items tested, B0008 proliferation was increased by a decrease in calcium choloride and an increase in the potassium chloride concentration. In the case of B00010 cells the optimum concentration of both these compounds is the same as that found in standard MCDB 104 medium. Those items which had altered optimum concentrations in B00010 cells were choline chloride, L-glutamine and L-proline. In all cases the optimum concentration was a reduction from the normal. All the other items tested, with exception of D-valine, were found

to have optimum concentrations similar to those found in the standard MCDB104 medium.

The concentration of D-valine present in the medium also has an effect on both cell lines. The optimum concentration for B0008 cells was 10 times that found in standard MCDB104 medium while for B00010 cells it was a ten fold decrease from the standard concentration. However, the level of cell growth achieved in the presence of D-valine is less than half that achieved in the presence of L-valine at its optimum concentration. This may be due to the absence of the enzyme D-amino acid oxidase from both cell lines. This is required to convert D-valine into the useful L-valine.

From this work it can be seen that the serum-free medium developed by us requires to be specifically tailor made for each of the two cell lines. For optimum cell growth of each cell line, the various components of the medium must be tested and optimised. Thus although some cell growth is achieved if B00010 cells are grown in the medium optimised for B0008 cells and vice versa, to achieve optimum cell growth of each melanoma cell line components must be optimised. Although only two human melanoma cell lines have been looked at it seems likely that the medium would have to be optimised for any other human melanoma cell lines looked at. However, it may be that if every component of MCDB104 was optimised, then a level of cell growth for both B0008 and B00010 cells may be achieved which would vary only slightly from that obtained if total optimisation for each component was carried out.

However, at present this is outwith the scope of this project.

7. Conclusions

1. From our experience the development of a universal serum-free medium for the optimum growth of all human melanoma cells is not at present practical.
2. To achieve an acceptable level of cell growth for all melanoma cells under serum-free conditions each component of the basic growth medium (MCDB104) must be optimised.
3. It is not at present possible to culture normal human melanocytes in the serum-free systems tested by us.
4. We found that we were unable to improve upon the two methods used for the culture of normal human melanocytes by either totally eliminating fibroblast contamination or increasing melanocyte numbers by further stimulation.

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