ANIMAL TISSUE CULTURE

Advances in Technique

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Edited by
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Preface

The development of cell culture and its application in the scientific field is based on first class techniques; no other standard is acceptable. A good theoretical knowledge will not on its own produce good cultures. It is the skill acquired by practice and, I believe, a sympathy for the subject that produces satisfactory results.

The purpose of this book is to bring together in one volume some of the developments in method which have taken place in recent years and are now used in cell culture. It is hoped that the information given will be of practical use to laboratory workers who are using cell cultures in their scientific work.

I would like to express my gratitude to all the contributors who have been most patient and helpful in the publication of this book.

Acknowledgements are due to the various organisations who have kindly given their permission to reproduce the various illustrations and photographs in this book.

G.D.W.

Contents

ONE

Mammalian cell culture media

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GENERAL CONSIDERATIONS

The design of media for cell culture creates two conflicting interests which are difficult to reconcile. The aim must be to provide an environment for the cell which is as close as possible to that experienced *in vivo*, yet in order to obtain some measure of control and standardisation over the conditions it is also necessary to define each of the constituents. Whereas natural media, body fluids, tissue extracts etc., fulfil the conditions of the former they fall far short of fulfilling the latter. Likewise, media made up of purified and characterised components bear little resemblance to natural conditions.

The problem is most easily solved for short-term survival, since the only factors of importance are osmotic pressure, hydrogen ion concentration, other inorganic ions, source of carbohydrate and gases. Combinations of this type are known as balanced salt solutions. The osmotic pressure of a normal mammalian cell is in the region of 7·6 atmospheres at 38°C corresponding to a depression of freezing point of about 0·63°C. Although cells are not greatly affected by changes in the osmotic pressure as large as ±10 per cent, or even greater providing the change takes place slowly, it is important to keep close to the normal. Sodium chloride makes the greatest contribution to the osmotic pressure in animal cells and in most cell culture media, with other inorganic ions and glucose also playing their part. High molecular weight substances make relatively little contribution. It is therefore easier, from the point of maintaining a stable osmotic pressure, to vary the concentration of a protein in the medium rather than that of a small molecule such as glucose.

The hydrogen ion concentration of medium must be kept close to neutrality, even though most tissues will tolerate quite wide divergencies from pH 7·0. Optimal growth normally occurs between pH 7·2 and 7·4 and for certain cells,

for example human diploid cell lines, it is important not to exceed these limits. Survival of most other tissues is not likely to persist much beyond pH 6·8 and 7·6. For many years the pH in culture media has been controlled by a buffer system modelled on the naturally occurring CO_2/ bicarbonate system present in blood plasma. The bicarbonate is added to the medium as part of the balanced salt solution, which normally includes a weak phosphate buffer. Although this system works quite satisfactorily and is used in the majority of culture media, it has a number of disadvantages. CO_2 is readily lost from the medium, both during storage and use, with a consequent rise in pH. In a closed culture vessel, cell respiration usually provides sufficient CO_2 and acid metabolites to maintain the pH at a satisfactory level. During the initiation of a culture, however, the pH can rise considerably. The problem may be overcome by bubbling CO_2 through the medium prior to use, or by incubating the cultures in open vessels in an incubator with a CO_2 enriched atmosphere.

In addition to these difficulties, the pKa of sodium bicarbonate is 6·1, and this results in suboptimal buffering over the physiological range. These disadvantages have stimulated a number of attempts to find a non-toxic, non-volatile buffer system. Glycylglycine[1] and *tris*-(hydroxymethyl) aminomethane[2] have been used for some time, although Martin[3] has more recently stressed the importance of adjusting the pH of *tris*- buffers with HCl rather than with the more toxic citric or malic acids. He found good proliferation of cells when this buffer was used in a simple chemically defined medium. Nevertheless, several workers have reported that both glycylglycine and *tris*- buffers are toxic over long periods and Shipman[4] claims that 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) has considerable advantages over them. HEPES is non-toxic in all systems tested so far, exhibits no metal binding and is readily soluble at 0°C. A wide variety of cells grew well in its presence without any effect on subsequent virus titrations or haemagglutination. In 1963, Leibowitz[5] devised a medium for use in free gas exchange with air. Free-base amino acids, especially L-arginine, L-cysteine and L-histidine, were substituted for sodium bicarbonate, while acid production was reduced by substituting more oxidised carbohydrate sources for glucose.

Other inorganic ions required for survival include sodium, potassium, calcium, magnesium, iron, carbonate, phosphate and sulphate. Their function, although not fully understood, includes maintenance of osmotic pressure, contribution to enzyme and metabolic activity and formation of the cell-to-glass bond. Glucose is the most common carbohydrate source and is a constituent of most balanced salt solutions. Some of the more complex media contain other sugars or simpler compounds including lactic, pyruvic and acetic acids in addition to, or instead of glucose. Oxygen and carbon dioxide are almost certainly essential for survival. For most cultures, dissolved oxygen in the medium is sufficient, but it is important to ensure that the ratio of air-space to medium in closed vessels is large enough to supply an adequate concentration. Carbon dioxide is produced by metabolism, so that its exclusion from medium and thus its requirement is difficult to establish. There is, however, strong evidence to suggest that some cells do have an absolute requirement.

Media become correspondingly more complex for long-term survival and growth. Balanced salt solutions will maintain cells in a healthy condition for

only a relatively short period, but this can be considerably extended by the addition of a cell protective agent such as serum, gelatin, albumin or methyl cellulose.[6] However, in order to develop the full potential and function of the cell, a much wider range of medium components is required so that new cellular material can be produced and metabolic activity increased. Amino acids are important among these. In addition to the ten 'essential' amino acids required by mammals, (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane and valine), mammalian cells in culture also require cystine and tyrosine[7]. Most cells, in particular transformed cells, also have a high requirement for glutamine[8]. These amino acids represent the bare minimum and the majority of cells will grow better in the presence of additional amino acids. For example, the growth requirements of single cells are more extensive than for larger cell populations. Lockart and Eagle[9] found that seven other amino acids, especially serine, had to be included in the medium to support the growth of single cells. Only L-isomers of amino acids are used by cells, but the presence of excess quantities of the D-isomers of the 'essential' amino acids has no effect. However, D-isomers of some non-essential amino acids may be inhibitory[10]. Cells contaminated with mycoplasma have an abnormally high requirement for arginine[11] and this may possibly explain the relatively high concentration of arginine in some media.

The majority of essential vitamins appear to be of the B group. They include *p*-aminobenzoic acid, biotin, choline, folic acid, nicotinic acid, pantothenic acid, pyridoxal, riboflavin, thiamin and inositol. Most of these are known to form integral parts of coenzymes involved in metabolism and hence some media include the coenzymes as well. Media designed to be used in the absence of serum also often include the fat soluble vitamins. Another important group of substances found in complex media are the reducing agents, glutathione, ascorbic acid and L-cysteine. Cells will use nucleic acid derivatives preferentially rather than synthesising nucleic acids directly from simpler molecules and thus these, too, are common constituents of defined media. The role of serum in medium is the subject of many conflicting reports. What is clear, however, is that in spite of highly sophisticated attempts to develop a serum-free medium, very few cells can be persuaded to grow in the absence of serum. Even when this is achieved, growth is always markedly improved by its addition.

A final group of medium additives comprising the antibiotics plays no part in cell nutrition or metabolism. All media used in cell culture readily support the growth of microbial contaminants. Antibiotics provide a useful adjunct to careful sterile technique for the prevention of contamination. There is no reason why they should not be used with reasonable discrimination, providing the potential dangers are fully understood. The main dangers are suppression rather than elimination of contaminants, and the creation of a false sense of security by the constant addition of antibiotics. When they are used routinely for cell lines, they should be withdrawn at intervals in order to unmask possible low grade contamination. It must also be remembered that some antibiotics are quite toxic at their effective concentrations and that some, for example mycostatin and penicillin, are relatively unstable. Thus it is possible for these to have decayed and become ineffective before the end of an experiment.

The most commonly used antibiotics are penicillin and streptomycin,

although dihydrostreptomycin is suggested as an alternative to the latter since it is as effective, but considerably less toxic at the same concentration[12]. Tomkins and Ferguson[13] proposed the following list as suitable for the elimination of most micro-organisms from cell cultures: kanamycin, neomycin, fungizone, chloramphenicol, viomycin and polymyxin B.

Mycoplasmas present a greater problem than most other organisms, because they are more difficult to detect and eliminate. Myo-crisin, which is slightly toxic, or tylosin tartrate[14], which can be used safely up to 100 μg/ml and is stable at +4°C, are used for the treatment of mycoplasmas. However, even these substances, in common with many tetracyclines tend to suppress rather than eradicate and should be used only when strictly necessary.

PRACTICAL CONSIDERATIONS

Many of the practical difficulties associated with the preparation of cell culture media have been alleviated by the appearance on the market of a wide range of fully tested media prepared from high quality reagents. For those wishing to undertake this work for themselves, the details of preparing complex media by means of stock concentrates of compatible components and the methods for obtaining various natural materials from living sources, have been fully described in a number of earlier treatises[15–17]. It is not proposed, therefore, to deal with this aspect, but rather consider some of the problems which still remain in association with commercial media.

Liquid medium presents few problems. It is generally supplied at working strength or as a tenfold concentrate. Working strength medium is usually supplied complete and only requires the addition of serum before use as a growth medium. Since it normally contains antibiotics, it is recommended that it be used fairly rapidly, and hence over a long period, many different batches would have to be used. Batches can vary slightly and thus it is impossible to guarantee exact replication of the conditions in each subsequent experiment. The same disadvantage applies to concentrated medium, but since sodium bicarbonate and the antibiotics are generally omitted, the medium can be used over longer periods. Complex defined media include a number of substances, for instance glutamine, which are relatively unstable in solution. These substances tend to be more stable at lower temperatures, hence medium should be kept at +4°C when not in use, or at −20°C for long-term storage.

As techniques in cell culture have improved, the need for highly standardised bulk media has increased. Great difficulties were experienced in propagating Hayflick's WI-38 cell line[18] when it was first distributed in Europe, and this was almost certainly due both to variations in purity of the components, and of the recipes followed in preparing the medium. Because of the limitations in the storage of liquid media, increasing attention has been paid to the preparation of media in a dry powdered form[19–21]. Dry medium, prepared either by ball-milling or by spray- or freeze-drying is now available from a number of commercial sources. Such media support the growth of cell cultures equally as well as media prepared in the traditional manner. Other advantages include lower costs, greater stability, uniformity of components by bulk buying of carefully selected

ingredients and low transit costs. Although it is recommended that powdered medium is stored at +4°C, it is stable for long periods at room temperatures and hence may be sent long distances without harmful effects. Ball-milled media suffer from the slight disadvantage that component particles can assume an uneven distribution during long storage periods. For this reason it is best to purchase the medium in preweighed aliquots in amounts that satisfy monthly or weekly requirements. Some manufacturers provide the additional service of releasing samples of batches of dry media so that customers can ensure its suitability for their purposes before committing themselves to the purchase of large quantities.

One of the most crucial stages in the preparation of a medium is the sterilisation of the final product. Heat stable media may be sterilised by autoclaving. This method is both cheap and, providing due attention is paid to the conditions, highly effective. Autoclaving can result in changes in the medium, but these need not necessarily be deleterious. Sargeant and Smith[22] reported that a compound was formed, when glucose and phosphate ions were autoclaved together, which stimulated growth and the attachment of epithelial cells to glass. It was also found[23] that maintenance of cultures was impaired when the growth medium was filtered instead of autoclaved. For media with labile components, and this includes the majority of chemically defined or semidefined media, effective sterility is usually achieved by filtration. However, it has been shown[24] that Eagle's Media can be rendered thermostable at pH 4–4·5 by the incorporation of a succinate buffer. These autoclavable media require the addition of glutamine and bicarbonate solutions prior to use. Most filtration methods have their attendant problems. Ceramic candles were once widely used, but tended to impair the growth promoting potential. This was probably caused either by selective adsorption of components from the medium or by catalytic activity occuring on the porous surface of the candle. Seitz filtration suffers from the presence of a toxic substance in the asbestos pads which is extracted by the passage of medium[25]. It is generally agreed that membrane filtration represents the best method, although some membranes are supplied impregnated with a detergent to enable wetting and facilitate sterilisation, and this may be cytotoxic[26]. As with asbestos pads, toxicity can be removed by prewashing. It is important to avoid the use of chromium plated apparatus since copper may be leached out from the underlying brass by acid filtrates, giving a toxic product.

A wide range of sera is now available to enrich growth media. These are generally supplied untreated or inactivated by heating to 56°C for 30 min. Heat inactivation destroys complement and some contaminating viruses, thus rendering cultures grown on it more useful for virological procedures. Serum treated in this way may have slightly diminished growth promoting properties, but can be stored satisfactorily at +4°C. Unheated sera are rather less stable and should be stored at −20°C. In all cases where media or medium components are stored frozen, attention should be paid to the type of container and the provision of adequate space for expansion during freezing.

All media offered for sale are tested by the manufacturers for their ability to support growth and for freedom from toxicity. There is, however, some divergence of opinion on the best way of doing this. Medium designed for the

growth of primary cultures is relatively easy to test. Cells which are sensitive to medium quality are used, and growth, morphology and evidence of toxicity in the test medium and in a standard control medium are carefully compared. The complexity of the test increases for medium designed for continuous cell culture. Two main possibilities exist, that is a plating efficiency test or a test involving a number of serial passages. Relative plating efficiency tests can certainly detect quite small differences in the quality of a medium, but it has been suggested[27] that high plating efficiency does not necessarily correlate with good growth promotion. This casts some doubt on the validity of plating efficiency as a method for assessing the growth potential of medium. Serial subcultivation tests are time consuming, but since the conditions of the test are the same as those under which the medium would normally be used, the results are probably more significant. It is generally found that if a medium will support the growth of a cell line for at least six subcultivations, thereafter the limit in the number of subcultivations, if any, is set by the cells themselves rather than by the medium. It is clear, therefore, that a medium should be tested over at least six serial subcultivations in order to be certain that it is suitable for the prolonged growth of cell lines.

BALANCED SALT SOLUTIONS

All balanced salt solutions (BSS) are developments from the physiological saline devised by Ringer[28]. His mixture consisted of the three cations, calcium, potassium and sodium in similar proportions to those existing in sea water or the blood of higher animals. Tyrode's solution[29] was the first BSS formulated specifically for supporting the metabolism of mammalian cells, but had the disadvantage that great care had to be taken during preparation to avoid calcium precipitation. Since then a number of BSS have appeared. None of these have any specific advantage so far as growth is concerned, but have been designed with improved buffering capacity or to prevent precipitation. The two most commonly used today are those of Earle[30] and Hanks[31]. Earle's BSS is more strongly buffered by virtue of a higher concentration of sodium bicarbonate. Hanks' BSS was primarily designed to equilibrate with air, rather than CO_2. It is particularly of use in conjunction with cells with which bicarbonate is toxic. Dulbecco and Vogt's phosphate buffered saline[32] is a valuable fluid for irrigating and washing cells and as a base for trypsin solutions. The composition of the BSS listed above is shown in *Table 1.1*. A more detailed account of the development of BSS and a description of lesser used specialist formulae is given in the review of Stewart and Kirk[33].

GROWTH MEDIA

NATURAL MEDIA

With the exception of serum, natural media are only rarely obtainable from commercial sources. This perhaps explains the current decline in their use for

Table 1.1 COMPOSITION OF BALANCED SALT SOLUTIONS (g/l)

	Phenol red	NaCl	KCl	$CaCl_2$ *Anhydrous*	$MgCl_2 \cdot 6H_2O$	$MgSO_4 \cdot 7H_2O$	$Na_2HPO_4 \cdot H_2O$	$NaH_2PO_4 \cdot 2H_2O$	KH_2PO_4	*Glucose*	$NaHCO_3$
Ringer	–	9·00	0·42	0·25	–	–	–	–	–	–	–
Tyrode	–	8·00	0·20	0·20	0·10	–	–	0·05	–	1·00	1·00
Earle	0·02	6·80	0·40	0·20	–	0·20	–	0·14	–	1·00	2·20
Hanks	0·02	8·00	0·40	0·14	–	0·20	0·06	–	0·06	1·00	0·35
Dulbecco and Vogt	0·02	8·00	0·20	0·10	0·10	–	1·42	–	0·20	–	–

until quite recently they were commonly employed. Thus in 1954, Malherbe[34] described a medium for the growth of human embryonic cells consisting of allantoic or amniotic fluid (90%) normal horse serum (5%) and bovine embryo extract (5%). In 1956 Jordan[35] used homologous human serum (35%), chick embryo extract (5%) and Hanks' BSS (60%) for the culture of human nasal cells. Plasma clot cultures[36], in which tissue is embedded in a coagulum caused by mixing chicken plasma and chick embryo extract, are still used when only small fragments of tissue are available to initiate a culture. It is possible to obtain freeze-dried or fresh chicken plasma in siliconised tubes for this purpose.

Apart from serum, the most frequently used medium ingredient from a natural source is lactalbumin hydrolysate. It is prepared by enzymic hydrolysis of a milk protein and is marketed by a number of companies. Melnick's medium[37] comprising Earle's BSS, lactalbumin hydrolysate and serum was originally designed for monkey kidney cell culture, but it provides an excellent growth medium for most primary cells. It must be pointed out, however, that commercial products can vary in colour, amino acid content and nutritional activity. It was found[38] that some cell lines lost their ability to attach to glass and that growth was poorer when batches were changed. The concentration of lactalbumin hydrolysate in the medium profoundly affects growth[39], with a sharp peak in optimum activity. As mentioned earlier[23], the method of sterilisation also markedly affects the performance of lactalbumin hydrolysate in the medium. A second protein digest which is a fairly frequent additive to culture medium, is tryptose phosphate broth[40]. It is particularly useful to enrich semi-defined growth media.

MEDIA COMPRISING BOTH NATURAL AND DEFINED COMPONENTS

Although natural media go part way to fulfilling the conditions described in the first paragraph of this chapter, they fall very far short of achieving the standardisation and control which is so important for current techniques. At the same time, completely defined media have two major defects. They support the growth of only a narrow range of highly adapted cells, and their performance is always improved by the addition of serum. Serum is, therefore, a constituent of the majority of growth media. Media have been designed in which only the components essential for cell growth have been added, but which require the addition of whole or dialysed serum. Probably the most frequently used media of this type were designed by Eagle[41, 42]. Eagle's media include the thirteen amino acids and seven vitamins found to be essential for growth dissolved in Earle's BSS (*Table 1.2*). A number of cells will grow in Eagle's medium supplemented with only albumin and inositol[43], although 5–10% serum is usually added.

Sera from a number of sources are used for cell culture. The most commonly used are bovine, but sera from horses, humans, rabbits and other sources are also used and can be obtained commercially. In general, the younger the animal, the better the growth provided by the serum. Hence serum from adult cattle tends to be poorer than that from young calves, whereas foetal calf serum is superior to both. In view of the tremendous importance of serum in cell culture

medium, it is perhaps surprising that its function is not more fully understood. Opinions range over a number of possibilities. It seems almost certain that low molecular weight nutrient substances, found in association with, or absorbed to serum proteins play an important part. Dialysis can remove the growth promoting activity of serum[42, 44, 45], but this can be restored by the addition of proteose peptone (0·1%)[46]. Activity is not restored by adding certain vitamins,

Table 1.2 COMPOSITION OF EAGLE'S MEDIA IN mg/l
(mM equivalents for amino acids given in brackets)

	Basal Medium (1955)	*Minimum Essential Medium* (1959)
Amino acids		
L-arginine HCl	21 (0·1)	126 (0·6)
L-cystine	12 (0·05)	24 (0·1)
L-glutamine	292 (2·0)	292 (2·0)
L-histidine HCl	9·5 (0·05)	38 (0·2)
L-isoleucine	26 (0·2)	52 (0·4)
L-leucine	26 (0·2)	52 (0·4)
L-lysine HCl	36 (0·2)	72 (0·4)
L-methionine	7·5 (0·05)	15 (0·1)
L-phenyl alanine	18 (0·1)	32 (0·2)
L-threonine	24 (0·2)	48 (0·4)
L-tryptophane	4 (0·02)	10 (0·05)
L-tyrosine	18 (0·1)	36 (0·2)
L-valine	24 (0·2)	48 (0·4)
Vitamins		
Choline chloride	1	1
Folic acid	1	1
Inositol	2*	2
Nicotinamide	1	1
Calcium pantothenate	1	1
Pyridoxal HCl	1	1
Riboflavin	0·1	0·1
Thiamine	1	1
Salts (Earle's BSS)		
NaCl	6800	6800
KCl	400	400
$CaCl_2$	200	200
$MgSO_4.7H_2O$	200	200
$NaH_2PO_4.2H_2O$	150	150
$NaHCO_3$	2000	2000
Glucose	1000	1000

Biotin (1·0 mg/l is also often included.
* Not included in Eagle's original formula, but often a component of commercial media.

nucleic acid derivatives or amino acids. This indicates that peptides may be involved and this is further substantiated by Piez *et al.*[47]. There are indications, however, that the amino acids adsorbed to protein do play a part, since it has been found that serum at high concentrations may provide amino acids at concentrations which can reach threshold levels for survival in an otherwise

deficient medium[48]. A further group of low molecular weight substances thought to be of importance are lipid sources[49–51].

High molecular weight fractions of serum are also thought to play a nutritive role[52]. Both albumin[53] and α and β globulins[54–56] have been implicated. However, at least one group claims that serum protein is not degraded or incorporated by cells[57]. High molecular weight fractions may, however, act in an entirely different manner. It is suggested that they may play a physical role, by protecting cells from mechanical damage[56, 58, 59], or by acting as detoxifying agents[60]. Norkin *et al.*[51] showed that albumin was most effective in medium if the majority of low molecular weight substances bound to it, were extracted. This released binding sites which could then take up toxic substances from the medium. A further example of the physical action of serum is its ability to promote the attachment and spreading of cells to the substrate, particularly to glass[56, 61, 62]. This may be brought about by an alteration of the surface charge of the glass[63, 64]. The nature of the serum fraction which causes this is not clear, but is almost certainly associated with the α-globulins[65]. It has been identified as the glycoprotein fetuin[66], but another protein not identical to fetuin has also been found[67]. More recently, Wallis *et al.*[68] postulated that the function of serum in growth media was to inhibit proteolytic enzymes. They suggested that serum is required to inactivate residual trypsin remaining after enzymic disaggregation, together with proteases synthesised subsequently by the cells. It was concluded that 'toxic' sera may simply have little or no anti-enzyme component and that 'inactivation' may result in lowering its concentration and hence the growth promoting property of the serum. Which of these various functions serum fulfils, or the order of their importance, is still to be determined. The considerable complexity of serum makes a full understanding difficult, but until this is achieved, the discovery of a completely effective substitute will inevitably be delayed.

DEFINED MEDIA

From the discussion on the role of serum it can be seen that there are a number of possible approaches to the problem of serum substitution in media. Following the theory that serum was acting as a protective agent, Katsuta *et al.*[58] proposed alginic acid, dextran and polyvinylpyrrolidone (PVP) as alternatives. They adapted a strain of HeLa to grow on a medium containing 0·1% PVP (average molecular weight, 7×10^5), lactalbumin hydrolysate (0·4%) and yeast extract (0·8%) in a BSS[59]. Purified proteins, in particular insulin sometimes with the addition of inert polymers, have also been used[49, 69, 70]. Two of these groups[49, 70] include lipid sources in their medium and the former demonstrated that the methyl esters of long chain unsaturated fatty acids stimulated growth, whereas long chain saturated and branched chain fatty acids did not. Michl[71] worked for several years on a flattening fraction derived from serum. He later found that it could be replaced by carbamyl phosphate[72] which when added to a synthetic medium, provided a good substitute for whole serum, especially when insulin was present. Hams' medium F.10 (*Table 1.3*)[73] was specially devised to support the growth cells when supplemented with purified proteins. Each of the

Table 1.3 COMPOSITION OF HAM'S NUTRIENT MIXTURE, F.10 IN mg/l (mM equivalents for amino acids given in brackets)

Amino Acids		*Vitamins*	
L-alanine	8·91 (0·1)	Biotin	0·02
L-arginine HCl	211·00 (1·0)	Calcium pantothenate	0·71
L-aspartic acid	13·30 (0·1)	Choline chloride	0·69
L-asparagine HCl	15·00 (0·1)	Folic acid	1·32
L-cysteine HCl	31·50 (0·2)	Inositol	0·54
L-glutamic acid	14·70 (0·1)	Nicotinamide	0·61
L-glutamine	146·20 (0·1)	Pyridoxine HCl	0·20
Glycine	7·51 (0·1)	Riboflavin	0·37
L-histidine HCl	21·00 (0·1)	Thiamine HCl	1·01
L-isoleucine	2·60 (0·02)	Thymidine	0·73
L-leucine	13·10 (0·1)	Vitamin B_{12}	1·36
L-lysine HCl	29·30 (0·1)		
L-methionine	4·48 (0·03)	*Nucleic Acid Derivative*	
L-phenyl alanine	4·96 (0·03)	Hypoxanthine	4·08
L-proline	11·50 (0·1)		
L-serine	10·50 (0·1)	*Lipid Source*	
L-threonine	3·57 (0·03)	Lipoic acid	0·20
L-tryptophane	0·60 (0·003)		
L-tyrosine	1·81 (0·01)	*Carbohydrate source other than Glucose*	
L-valine	3·50 (0·03)	Sodium pyruvate	110·0
		Salts	
		$FeSo_4 . 7H_2O$	1·52
		$CuSO_4 . 5H_2O$	0·004
		$ZnSO_4 . 7H_2O$	0·05
		NaCl	7400·00
		KCl	285·00
		Na_2HPO_4	153·60
		KH_2PO_4	83·00
		$MgSO_4 . 7H_2O$	152·80
		$CaCl_2$	33·30
		$NaHCO_3$	1200·00
		Glucose	1100·00

components was added at an experimentally determined optimum concentration. It was designed to be used either with serum albumin and fetuin or with low concentrations of serum.

For some time the goal of many cell biologists has been to produce a chemically defined cell culture medium free from added protein. As early as 1911 Lewis and Lewis[74] attempted to do this when they found that glucose or amino acids and peptides prolonged the survival of chick embryo tissues in saline solutions. Modern attempts to solve the problem stem from 1950 and the Medium 199 of Morgan, Morton and Parker[75] (*Table 1.4*). Medium 199 included almost all the amino acids, vitamins, several nucleic acid derivatives, accessory growth factors, lipid sources and Earle's BSS supplemented with ferric nitrate. This exceedingly complex medium is still widely used as a maintenance medium and when supplemented with serum, as a growth medium. In the complete absence of serum, however, it is only able to maintain cells for relatively short periods. The medium was later modified[76] to give Medium 858. This was considerably more efficient that Medium 199. In particular it restored

Table 1.4 COMPOSITION OF SYNTHETIC MIXTURE NO. 199 IN mg/l
(mM equivalents for amino acids are given in brackets)

Amino Acids		*Coenzymes*	
L-alanine*	25·0 (0·30)	A.T.P.	10·0
L-arginine HCl	70·0 (0·40)		
L-aspartic acid*	30·0 (0·25)		
L-cystine	20·0 (0·75)	*Reducing Agents*	
L-glutamic acid*	75·0 (1·00)	Ascorbic acid	0·05
L-glutamine	100·0 (0·07)	L-cysteine HCl	0·1
Glycine	50·0 (0·70)	Glutathione	0·05
L-histidine HCl	20·0 (0·10)		
L-hydroxyproline	10·0 (0·75)		
L-isoleucine*	20·0 (0·15)	*Nucleic acid derivatives*	
L-leucine*	60·0 (0·45)	Adenine	10·0
L-lysine	70·0 (0·20)	Guanine	0·3
L-methionine*	15·0 (0·20)	Hypoxanthine	0·3
L-phenylalanine*	25·0 (0·15)	Thymine	0·3
L-proline	40·0 (0·35)	Uracil	0·3
L-serine*	25·0 (0·25)	Xanthine	0·3
L-threonine*	30·0 (0·25)	Adenylic acid	0·2
L-tryptophane*	10·0 (0·005)		
L-tyrosine	40·0 (0·20)		
L-valine*	25·0 (0·20)	*Lipid source*	
		Cholesterol	0·2
		Tween 80	5·0
Vitamins			
p-aminobenzoic acid	0·05		
Biotin	0·01	*Carbohydrate sources other than glucose*	
Calcium pantothenate	0·01	2-deoxy-D-ribose	0·5
Choline chloride	0·5	D-ribose	0·5
Folic acid	0·01	Sodium acetate	50·0
Inositol	0·05		
Niacin	0·025		
Nicotinamide	0·025	*Salts*	
Pyridoxal HCl	0·025	NaCl	6800·0
Pyridoxine HCl	0·025	KCl	400·0
Riboflavin	0·01	$CaCl_2$	200·0
Thiamine HCl	0·01	$MgSO_4 . 7H_2O$	200·0
Vitamin A	0·10	NaH_2PO_4	140·0
Vitamin D	0·10	$NaHCO_3$	2200·0
Vitamin E	0·01	$Fe(NO_3)_3 . 9H_2O$	0·1
Vitamin K	0·01		
		Glucose	1000·0

* Double quantity of DL amino acid in original formula.

the abnormally high oxidation potential of Medium 199 to nearer physiological levels by large increases in the amounts of the reducing agents, L-cysteine HCl, ascorbic acid and glutathione. Five B vitamins, thiamine, riboflavin, niacin, niacinamide and pantothenate were omitted because they were constituents of six coenzymes which were added. Two years later, Medium 858 was modified to give CMRL-1066 Medium[77]. Four fat soluble vitamins, A, D, E, and K were replaced by the five B vitamins previously left out. This medium, the result of