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Arthropod Cell Culture Systems

Edited by

Karl Maramorosch, Arthur H. Mcintosh

CRC Press
Taylor & Francis Group

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CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

First published 1994 by CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Reissued 2018 by CRC Press

Library of Congress Cataloging-in-Publication Data

Arthropod cell culture systems / edited by Karl Maramorosch, Arthur H.
McIntosh.
p. cm.
Includes bibliographical references (p.) and index.
ISBN 0-8493-7642-4
1. Arthropoda—Cytology. 2. Cell culture. I. Maramorosch, Karl.
II. McIntosh, Arthur H.
QL434.715.A78 1994
595' .2'0724—dc20 93-38285

A Library of Congress record exists under LC control number: 93038285

ISBN 13: 978-1-315-89080-7 (hbk)
ISBN 13: 978-1-351-06990-8 (ebk)

Visit the Taylor & Francis Web site at http://www.taylorandfrancis.com and the
CRC Press Web site at http://www.crcpress.com

PREFACE

The use of arthropod cell culture systems has increased rapidly over the past decade, assuming a prominent role in basic biological research and in biotechnology. Cell lines derived from invertebrate hosts are currently employed in studies of various biological processes in biochemistry, genetics, endocrinology, molecular biology, physiology, virology, medicine, and agriculture. Cultured arthropod cells can support the growth and replication of viruses, bacteria, and intracellular parasites, providing an important system for studying pathogens affecting plants, animals, and humans. Insect cell culture has provided a major impetus toward the commercial application of these systems in biotechnology and insect pest control. Numerous important contributions have been made employing arthropod cell culture systems but the information has been scattered in different journals. Therefore the editors felt that there remained an obvious need for a comprehensive book that would bring together the current research advances and describe the methods and diverse applications of arthropod cell culture.

This book brings together for the first time in two decades the large body of information and the significant achievements accomplished in laboratories throughout the world. The information has been presented by foremost authorities and pioneers of insect and tick cell culture, who contributed basic and applied concepts. Their international experience has been combined so that the latest developments in this fascinating and rapidly expanding field are presented in a comprehensive manner. The large body of information brings into sharp focus anticipated new directions in this field.

Specialists and pioneers from the United States, Japan, Switzerland, Slovakia and China have been invited to write chapters for this treatise based on their outstanding and authoritative knowledge of the subjects and their original contributions. This has been done with the intention to provide readers with a well balanced cross-section of current developments and to focus attention on such topics as preparation of cell culture media, cultivation of mosquito, lepidopteran, grasshopper and tick cells, the application of such cells to mammalian and plant virus research, and diverse applications in medicine, biology and agriculture. Significant strides have been made in Japan and a separate chapter has been devoted to work of Japanese cell culture pioneers.

The growing interest in insect and tick cell cultivation is closely linked with the worldwide growth of biotechnology. The editors feel that the combined efforts of eminent contributors to present new information will benefit all who are interested in arthropod cell systems. All chapters are well documented by tables, photographs, and up-to-date bibliographies. This book will be of interest to microbiologists, molecular biologists, parasitologists, virologists, entomologists, plant pathologists, geneticists and medical and agricultural researchers. It is hoped that this book will set the benchmark for future research and serve for years to come as a standard source of information for advanced research workers and students.

The editors express their sincere gratitude to the contributors for their effort and care with which they have prepared their chapters and to CRC Press for their part in indexing, proof-reading, and other aspects of production of this volume.

Karl Maramorosch
Arthur H. McIntosh

THE EDITORS

Karl Maramorosch, Ph.D., is Robert L. Starkey Professor of Microbiology and Professor of Entomology at Rutgers — The State University of New Jersey, New Brunswick, New Jersey.

Dr. Maramorosch, a native of Vienna, Austria, graduated from the Agricultural University of Warsaw, summa cum laude, and obtained his Ph.D. in 1949 from Columbia University. His scientific career began at Rockefeller University where he worked on plant viruses and insect vectors for 12 years. Later, he became Program Director of Virology and Insect Physiology at the Boyce Thompson Institute, and since 1974, he has been Professor II (Distinguished Professor) at Rutgers University, where he became the Robert L. Starkey Professor in 1983.

Dr. Maramorosch is a Fellow and former Recording Secretary and Vice-President of the New York Academy of Sciences, Fellow of the American Association for Advancement of Science, the Entomological Society of America, and the American Phytopathological Society, President of the International Association for Medicinal Forest Plants, Honorary Fellow of the Indian Virological Society, and a member of the Tissue Culture Association, Harvey Society, Society for Invertebrate Pathology, American Society for Microbiology, Electron Microscopy Society, and several other professional organizations. Dr. Maramorosch has been the recipient of grants from the National Science Foundation, National Institutes of Health, U.S. Department of Agriculture, Rockefeller Foundation, and private industry. He won the 1980 Wolf Prize in Agriculture and many other awards, including the Ciba Geigy Award in Entomology, Jurzykowski Award in Biology, AIBS Award of Distinction, Cressy Morisson Prize of the N.Y. Academy of Sciences, Waksman Award, and AAAS-Campbell Award. He was nominated by the Entomological Society of America for the National Medal of Science in 1983 and was the Founders Lecturer of the Society for Invertebrate Pathology in 1990. He is an elected member of the Leopoldina, oldest European Academy of Sciences, and a Fellow of the Indian National Academy of Sciences.

Dr. Maramorosch served twice as visiting Fulbright Professor in Yugoslavia and as visiting professor in the Peoples Republic of China, U.S.S.R., The Netherlands, Germany, Japan, India, and Poland. He was consultant to the Food and Agriculture Organization in the Philippines, India, Nigeria, Thailand, Sri Lanka, and Mauritius, and has held short-term assignments from the Rockefeller Foundation, the Agency for International Development, U.S. Department of Agriculture (FERRO), and Ford Foundation in Mexico, India, Kenya, and the Philippines. Dr. Maramorosch has edited more than 50 volumes on viruses, vectors, plant diseases, and tissue culture, and is the author or co-author of more than 600 research papers. His major research interests include comparative virology, invertebrate cell culture, parasitology, diseases caused by spirochetes, viroids, and spiroplasmas, biotechnology, and international scientific cooperation.

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Dr. McIntosh graduate in 1959 from McMaster University, Hamilton, Ontario, Canada with a B.A. He attended the University of Guelph, in Guelph, Ontario, Canada from 1960–1962 and graduated from the Department of Microbiology with an M.S. Dr. McIntosh worked on his doctorate from 1965–1969 in the Department of Microbiology, Harvard University School of Public Health and graduated in 1969. He then completed postdoctoral studies at Stanford Research Institute from 1969–1971 and accepted a position at Boyce Thompson Institute with Dr. Karl Maramorosch in Insect Virology and Cell Culture from 1971–1974. He was an Assistant Research Professor at Waksman Institute of Microbiology, Rutgers, Piscataway from 1974–1979 before joining the USDA-ARS. He holds a joint appointment in the Department

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Dr. McIntosh conducts research in the area of Biological Control utilizing insect cell culture to study the biological characteristics of baculoviruses. His interest also includes the study of the specificity of insect viruses. He has published over 60 research papers, reviews and book chapters.

Dr. McIntosh is a member of the Tissue Culture Association, Society for Invertebrate Pathology, Entomological Society of America, Japanese Society of Applied Entomology and Zoology and Sigma Xi.

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Chapter 1

INSECT CELL CULTURE MEDIA

Jun Mitsuhashi

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0-8493-7642-4/94/$0.00+$.50

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I. INTRODUCTION

Since Grace[1] established the first insect cell line, very many cell lines have been established during the last 3 decades from various insects. In order to maintain these cell lines, various culture media have been used, because there is no single culture medium that could maintain the growth of every insect cell. Some cell lines can be maintained in relatively nutrient-poor media but others cannot. Formulation of widely usable media is one of the tasks in developing new culture media.

II. HISTORY OF MEDIUM DEVELOPMENT

When the first trial to culture insect tissues *in vitro* was made, insect hemolymph itself was used as the culture medium by Goldschmidt.[2] At present, we know that hemolymph itself is not suitable for culture media, because it soon turns dark due to the formation of melanin by the action of phenol oxidase, and the resulting melanin is toxic to the cultured tissues or cells. Goldschmidt did not mention how he could overcome this sort of trouble. In the early stage of insect tissue cultures, most researchers used insect hemolymph or simple saline solutions as culture media, and the results were always short survival of tissues or cells. Trager[3] formulated a culture medium by combining inorganic salts, egg albumin digest, maltose, and insect hemolymph (Table 1). This medium was the first medium formulated specifically for insect cell growth and it supported growth for a considerable time. After Trager's work, several investigators examined the ability of vertebrate cell culture media to support insect cell growth although none of them gave promising results. Wyatt[4] formulated a synthetic culture medium by combining 6 inorganic salts, 21 amino acids, 4 other organic acids, and 3 sugars (Table 2). Her formulation was based on the chemical analyses of hemolymph from several insect species.[5] Although the medium could not support cell growth by itself, it supported the cell growth well when it was fortified by the addition of heat-treated insect hemolymph. This medium actually gave a clue to the formulation of insect cell culture media, and even at present we are using various modifications of her medium. Wyatt's medium was improved by Grace,[1] and with this medium, he established the first insect continuous cell lines. His modification was the addition of ten water-soluble vitamins, change of balance of inorganic salts, and increment of sucrose (Table 3). Grace's medium is well known as the medium which can support growth of various insect tissues, when supplemented with insect hemolymph or vertebrate sera, especially fetal bovine serum (FBS), which can be purchased commercially at present. Another type of culture medium is a chemically undefined medium whose major components are natural substances. Mitsuhashi and Maramorosch[6] formulated a simple medium presently called the MM medium. This medium contained lactalbumin hydrolysate, TC-yeastolate, and FBS as natural substances (Table 4). Originally this medium was developed for culturing leafhopper cells. It turned out later that this medium was well suited for mosquito cell cultures and also for culturing cells from various other insects, belonging to many orders. Furthermore, this medium is now known to support growth of many cell lines without sera supplements.[7]

In recent years, serum-free culture media were developed by many investigators. Now many insect cell lines can be cultured in serum-free media, but usually their growth rates are inferior in serum-free media, compared to serum-containing media. Some media sold commercially are said to maintain cell growth at the same rate as serum-containing media. However, these media do not necessarily support cell growth of every cell line. Most serum-free media contain more or less chemically undefined substances. It is, therefore, required to formulate completely chemically defined media. Once such a chemically defined medium was formulated,[8] however, this medium was rather difficult to prepare and it could support only limited cell line species (Table 5). Some media sold by industries are said to be

TABLE 1
Trager's Medium

Components	Amount[a]	Components	Amount[a]
NaCl	7.6	$CaCl_2$	11.1
$NaH_2PO_4 \cdot H_2O$	20.7	Maltose	2162.3
K_2HPO_4	20.4	Egg albumin digest	0.00137
$MgCl_2 \cdot 6H_2O$	20.3		

[a] Mol/l. pH 6.7. Osmolarity: –0.290°C (as freezing point depression). Used by adding 10% *Bombyx mori* hemolymph.

From Trager, W., *J. Exp. Med.*, 61, 501–513, 1935. With permission.

TABLE 2
Wyatt's Medium

Components	mg/100 ml	Components	mg/100 ml
NaH_2PO_4	110.0	Cysteine	8.0
KCl	298.0	L-Cystine	2.5
$MgCl_2 \cdot 6H_2O$	304.0	L-Glutamic acid	60.0
$MgSO_4 \cdot 7H_2O$	370.0	L-Glutamine	60.0
$CaCl_2$	81.0	Glycine	65.0
Glucose	70.0	L-Histidine	250.0
Fructose	40.0	DL-Isoleucine	10.0
Sucrose	40.0	DL-Leucine	15.0
Malic acid	67.0	DL-Lysine	125.0
α-Ketoglutaric acid	37.0	DL-Methionine	10.0
Succinic acid	6.0	L-Phenylalanine	15.0
Fumaric acid	5.5	L-Proline	35.0
DL-α-Alanine	45.0	DL-Serine	110.0
β-Alanine	20.0	DL-Threonine	35.0
L-Arginine	70.0	L-Tryptophan	10.0
L-Asparagine	35.0	L-Tyrosine	5.0
L-Aspartic acid	35.0	DL-Valine	20.0

Note: pH: 6.35. Osmolarity: –0.53°C (as freezing point depression). Used by adding heat-treated *Bombyx mori* hemolymph.

From Wyatt, S.S., *J. Gen. Physiol.*, 39, 841–852, 1956. With permission.

almost chemically defined, but they cannot be used for biochemical studies of cultured cells because the formulation has not been disclosed.

Recent development of biotechnology using insect cell cultures requires large-scale cultures of insect cells. Developments of low cost media are desired for large-scale cultures of insect cells especially for the purpose of production of viral insecticides. By the development of serum-free media, the cost of media has decreased considerably and now we can purchase fairly low-cost media, but still further cost reduction is needed.

III. MEDIUM COMPONENTS

In general, the main ingredients of culture media consist of inorganic salts, proteins, sugars as an energy source, and vitamins.

TABLE 3
Grace's Medium

Components	mg/100 ml	Components	mg/100 ml
$NaH_2PO_4 \cdot 2H_2O$	114.0	L-Threonine	17.5
$NaHCO_3$	35.0	L-Tryptophan	10.0
KCl	224.0	L-Tyrosine	5.0
$MgCl_2 \cdot 6H_2O$	228.0	L-Valine	10.0
$MgSO_4 \cdot 7H_2O$	278.0	Sucrose	2268.0
$CaCl_2$	100.0	Fructose	40.0
L-α-Alanine	22.5	Glucose	70.0
β-Alanine	20.0	Malic acid	67.0
L-Arginine·HCl	70.0	α-Ketoglutaric acid	37.0
L-Asparagine	35.0	Succinic acid	6.0
L-Aspartic acid	35.0	Fumaric acid	5.5
L-Cystine·HCl	70.0	Thiamine·HCl	0.002
L-Glutamic acid	60.0	Riboflavin	0.002
L-Glutamine	60.0	Calcium pantothenate	0.002
Glycine	65.0	Pyridoxine·HCl	0.002
L-Histidine	250.0	*p*-Aminobenzoic acid	0.002
L-Isoleucine	5.0	Folic acid	0.002
L-Leucine	7.5	Niacin	0.002
L-Lysine·HCl	62.5	Isoinositol	0.002
L-Methionine	5.0	Biotin	0.001
L-Phenylalanine	15.0	Choline chloride	0.02
L-Proline	35.0	Penicillin G, sodium salt	3.0
DL-Serine	110.0	Streptomycin sulfate	10.0

Note: pH: 6.5. Used by adding 3% heat-treated hemolymph of *Antheraea pernyi* or 1% of the hemolymph with 1% bovine plasma albumin fraction V.

From Grace, T.D.C., *Nature,* 195, 788–789, 1962. With permission.

TABLE 4
Mitsuhashi and Maramorosch's MM Medium

Components	mg/100 ml	Components	mg/100 ml
NaCl	700.0	D-Glucose	400.0
$NaH_2PO_4 \cdot H_2O$	20.0	Lactalbumin hydrolysate	650.0
$NaHCO_3$	12.0	TC-Yeastolate	500.0
KCl	20.0	Fetal bovine serum	0–20 ml
$MgCl_2 \cdot 6H_2O$	10.0		
$CaCl_2 \cdot 2H_2O$	20.0		

Note: pH: 6.5. Osmolarity: 413 mOsm/kg.

From Mitsuhashi, J. and Maramorosch, K., *Contrib. Boyce Thompson Inst.,* 22, 435–460, 1964. With permission.

Insect cells are said to be insensitive to ion balance and change of pH. Most media contain potassium chloride, sodium phosphate, sodium bicarbonate, magnesium chloride, and calcium chloride. Some media also contain sodium chloride or magnesium sulfate. Sodium chloride is not important for insect cells probably because insect hemolymph contains only a little sodium chloride. Since most insect cells are tolerant to a wide range of pH, incorporation of

TABLE 5
Wilkie et al.'s CDM (Chemically Defined Medium)

Components	mg/100 ml	Components	mg/100 ml
$NaH_2PO_4 \cdot 2H_2O$	114.0	Arachidonic acid	0.002
$NaHCO_3$	35.0	Putrescine	0.1
KCl	287.0	Spermidine	0.1
$MgCl_2 \cdot 6H_2O$	228.0	Spermine·4HCl	0.1
$MgSO_4 \cdot 7H_2O$	278.0	Carnitine	0.1
$CaCl_2 \cdot 2H_2O$	99.7	α-Amino-*n*-butyric acid	0.1
L-α-Alanine	22.5	*o*-Phosphorylethanolamine	0.2
L-Arginine	55.0	Taurine	0.1
L-Asparagine	35.0	Riboflavin	0.02
L-Asparate (K^+ salt)	45.0	*p*-Aminobenzoic acid	0.2
L-Glutamate (K^+ salt)·H_2O	82.9	Folic acid	0.1
L-Glutamine	60.0	D-Biotin	0.005
Glycine	65.0	Calcium D-pantothenate	0.12
L-Histidine·HCl·H_2O	338.0	Isoinositol	0.2
L-Isoleucine	5.0	Ascorbic acid	0.02
L-Leucine	7.5	Cyanocobalamin	0.1
L-Lysine·HCl	62.5	Nicotinamide	0.12
L-Methionine	5.0	Thiamine·HCl	0.2
L-Phenylalanine	15.0	Pyridoxine·HCl	0.1
L-Proline	35.0	Choline chloride	2.0
L-Serine	55.0	Hypoxanthine	1.0
L-Threonine	17.5	$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$	0.5
L-Tryptophan	10.0	$ZnSO_4 \cdot 7H_2O$	0.044
L-Tyrosine	7.0	$CuSO_4 \cdot 5H_2O$	0.039
L-Valine	10.0	$MnCl_2 \cdot 4H_2O$	0.035
L-Cystine	7.5	Trilinolein	0.01
α-D-Glucose	400.0	Trilinolenin	0.01
Methylcellulose (15 cps)	200.0	Phosphatidylcholine	0.02
Stearic acid	0.01	α-Tocopherol acetate	0.001
Myristic acid	0.01	Cholesterol	0.1
Oleic acid	0.01	β-Sitosterol	0.1
Linoleic acid	0.01	Stigmasterol	0.1
Linolenic acid	0.01	Tween 80	2.0
Palmitic acid	0.01	Ethanol	0.2 ml
Palmitoleic acid	0.01		

Note: pH: 6.3. Osmolarity: 330 mOsm/kg. Used as a serum-free medium.

From Wilkie, G.E.I., Stockdale, H., and Pirt, S.V., *Dev. Biol. Standard.*, 46, 29–37, 1980. With permission.

buffer to inorganic salt composition is not common, although some media contain some buffers, such as tris buffer,[9] glycine buffer,[10] and so on. There are two types of inorganic salt compositions, one that contains about 0.8% sodium chloride and is similar to vertebrate Ringer solution, the other that does not contain sodium chloride and instead contains potassium chloride or magnesium chloride as main components. Many cell lines can be cultured in media based on either type of inorganic salt composition.

As a protein source, usually combinations of free amino acids are used. The amino acid mixture consists of 20 amino acids, which are constituents of protein, and β-alanine. In vertebrate cell culture, β-alanine has never been a component of culture media. In insect cell cultures, it has been incorporated since Wyatt's medium,[3] because insect hemolymph reportedly contains considerable amounts of β-alanine. It turned out later, however, that β-alanine was not necessary or was even detrimental for the growth of insect cells.[11] Based on this

finding, some recently developed media omitted β-alanine from the amino acid mixture. Among 20 amino acids, arginine, cysteine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine are essential for many insect cell lines. However, nutritional requirements are different for different cell lines. For example, glutamine is essential for many insect cell lines, but some cell lines can grow without glutamine and even seem to synthesize glutamine from glutamic acid and ammonia.[12]

As for sugars, glucose is sufficient to provide the necessary energy source. Fructose, trehalose, and maltose can be utilized by insect cells. Generally sucrose is not considered to be utilizable, but some cell lines definitely can utilize it.[13,14] Moreover, many insect cell lines have α-glucosidase activity which digests sucrose.[15,16]

For vitamins, usually ten vitamins of the B group are used. They are thiamine, riboflavin, calcium pantothenate, *p*-aminobenzoic acid, biotin, inositol, pyridoxine, nicotinic acid, folic acid, and choline chloride. Some media contain vitamins A, B_{12}, C, and E. However, their roles are not clear.

Conventional media are often constructed with chemically undefined natural substances. Instead of an amino acid mixture, lactalbumin hydrolysate has been used frequently. Casein hydrolysate, egg albumin digest, or bactopeptone are also used for this purpose. As vitamin sources, yeast extracts, especially TC-yeastolate (Difco, Detroit, MI), are common. Sea water may be used in place of the inorganic salt mixture.[17]

In addition to the above basal part of culture media, the media are usually fortified by adding some cell growth-promoting substances. As such a substance, insect hemolymph has been used since the early stages of insect cell cultures. Insect hemolymph is, however, difficult to obtain in large quantities. Therefore, FBS has been widely used since it proved to promote insect cell growth.[6] After development of serum-free culture media, lipid mixture, yeast extract, cod liver oil, egg yolk, and so on have been used instead of sera.

The compositions of some representative culture media are shown in Tables 6 to 10.

IV. PREPARATION OF MEDIA

Simple media can be prepared directly according to the formulae. An example of such a medium is the MM medium (Table 4) with or without serum. Sera supplied from industries are sterile. Therefore, sera can be added under sterile conditions after media are sterilized. Even a simple medium, if prepared frequently, can easily be prepared from concentrated stock solutions of major parts of the medium. For instance, the salts in the MM medium can be stored as two separate, ten times concentrated, stock solutions. The one is the solution of $NaHCO_3$ and the other is the mixture of the remaining five inorganic salts (Table 4). When the complete medium is prepared, equal parts of both salt stock solutions are added to a 5-times volume of distilled water, and then lactalbumin hydrolysate, TC-yeastolate, and glucose are dissolved in it.

In order to prepare a complex culture medium such as Grace's medium (Table 3) and the like, it is recommended to prepare it by dividing the ingredients into several groups. Hereafter, the method for preparing MGM-450 medium (Table 10) is described as an example.

A. PREPARATION OF STOCK SOLUTIONS

1. Stock Solutions of Inorganic Salts (ISM-A and ISM-B)

These stock solutions are ten times concentrated mixtures of inorganic salts. In order to prepare 1 l stock solution, dissolve 2.92 g $NaHCO_3$ and 231.33 g $MgSO_4 \cdot 7H_2O$ in about 900 ml distilled water, and bring up the solution to 1 l by adding distilled water. This is ISM-A. Likewise, dissolve 9.58 g $NaH_2PO_4 \cdot 2H_2O$, 8.75 g KCl, 19.1 g $MgCl_2 \cdot 6H_2O$, and 8.33 g $CaCl_2$ in about 900 ml distilled water and adjust the volume to 1 l with distilled water. This is ISM-B. Both solutions can be stored in a refrigerator for several months.

TABLE 6
Weiss et al.'s IPL-41 Medium

Components	mg/100 ml	Components	mg/100 ml
$NaH_2PO_4 \cdot H_2O$	116.0	L-Valine	50.0
$NaHCO_3$	35.0	Sucrose	1650.0
KCl	120.0	Glucose	250.0
$CaCl_2$	50.0	Maltose	100.0
$MgSO_4 \cdot 7H_2O$	188.0	Malic acid	5.36
β-Alanine	30.0	α-Ketoglutaric acid	2.96
L-Arginine·HCl	80.0	Succinic acid	0.48
L-Asparagine	130.0	Fumaric acid	0.44
L-Aspartic acid	130.0	Thiamine·HCl	0.008
L-Cystine	10.0	Riboflavin	0.008
L-Glutamic acid	150.0	Calcium pantothenate	0.0008
L-Glutamine	100.0	Pyridoxine·HCl	0.04
Glycine	20.0	*p*-Aminobenzoic acid	0.032
L-Histidine	20.0	Folic acid	0.008
Hydroxy-L-proline	80.0	Niacin	0.016
L-Isoleucine	75.0	Isoinositol	0.04
L-Leucine	25.0	Biotin	0.016
L-Lysine·HCl	70.0	Cyanocobalamin	0.024
L-Methionine	100.0	Choline chloride	2.0
L-Proline	50.0	$ZnCl_2$	0.004
L-Phenylalanine	100.0	$MnCl_2 \cdot 4H_2O$	0.002
DL-Serine	40.0	$CuCl_2 \cdot 2H_2O$	0.02
L-Threonine	20.0	$(NH_4)Mo_7O_{24} \cdot 4H_2O$	0.004
L-Tryptophan	10.0	$CoCl_2 \cdot 6H_2O$	0.005
L-Tyrosine	25.0	$FeSO_4 \cdot 7H_2O$	0.055

Note: Osmolarity: 360–375 mOsm/kg. Additives: Fetal bovine serum 10%.

From Weiss, S.A. et al., *In Vitro*, 17, 495–502, 1981. With permission.

TABLE 7
Echalier and Ohanessian's D-20 Medium

Components	mg/100 ml	Components	mg/100 ml
$NaH_2PO_4 \cdot 2H_2O$	47.0	Thiamine·HCl	0.002
$MgCl_2 \cdot 6H_2O$	100.0	Riboflavin	0.002
$MgSO_4 \cdot 7H_2O$	370.0	Pyridoxine	0.002
$CaCl_2$	89.0	Niacin	0.002
Glucose	200.0	Calcium pantothenate	0.002
Glutamine[a]	1087.8	Biotin	0.001
Glycine[a]	553.8	Folic acid	0.002
Malic acid	67.0	Inositol	0.002
Succinic acid	6.0	*p*-Aminobenzoic acid	0.002
Sodium acetate·$3H_2O$	2.5	Choline chloride	0.02
Lactalbumin hydrolysate	1500.0	TC-yeastolate	150.0

Note: pH: 6.7. Osmolarity: –0.66°C (as freezing point depression). Additives: Fetal bovine serum 10–20%.

[a] Used as sodium glutamate, sodium glycinate, potassium glutamate, and potassium glycinate.

From Echalier, G. and Ohanessian, A., *In Vitro*, 6, 162–172, 1970. With permission.

TABLE 8
Shield and Sang's M3 Medium

Components	mg/100 ml	Components	mg/100 ml
$MgSO_4 \cdot 7H_2O$	440.0	Glycine	50.0
$CaCl_2 \cdot 6H_2O$	150.0	α-Alanine	150.0
Potassium glutamate·H_2O	788.0	Valine	40.0
Sodium glutamate	653.0	Methionine	25.0
$NaH_2PO_4 \cdot 2H_2O$	88.0	Isoleucine	25.0
$KHCO_3$	50.0	Leucine	40.0
Glucose	1000.0	Tyrosine	25.0
Oxaloacetic acid	25.0	Phenylalanine	25.0
BIS-TRIS	105.0	β-Alanine	25.0
TC-yeastolate	100.0	Histidine	55.0
Aspartic acid	30.0	Tryptophan	10.0
Threonine	50.0	Arginine	50.0
Serine	35.0	Lysine·HCl	85.0
Asparagine	30.0	Cysteine·HCl	20.0
Glutamine	60.0	Choline chloride	5.0
Proline	40.0		

Note: pH: 6.8. Additives: Fetal bovine serum 10%.

From Shields, G. and Sang, J.H., *Drosophila Information Service*, 52, 161, 1977. With permission.

TABLE 9
Maiorella et al.'s Medium

Components	Amount
IPL-41 medium	1000 ml
Cod liver fatty acid methyl ester	10.0 mg
Tween 80	25.0 mg
Cholesterol	4.5 mg
α-Tocopherol acetate	2.0 mg
Ethyl alcohol	1.0 ml
10% aqueous solution of pluronic polyol F-68	10.0 ml

Note: Used as serum-free medium.

From Maiorella, B. et al., *Bio/Technology*, 6, 1406–1410, 1988. With permission.

2. Stock Solution of Amino Acids, Sugars, and Organic Acids (ASO)

In order to prepare 1 l of the ASO, dissolve 105 mg L-cystine and 210 mg L-tyrosine in 20 ml of 1 *N* HCl. When both amino acids have dissolved completely, add distilled water up to 900 ml. Then dissolve the following, one by one: 1,315 mg L-α-alanine, 835 mg β-alanine, 2,915 mg L-arginine hydrochloride, 1,465 mg L-aspartic acid, 1,465 mg L-asparagine, 2,500 mg L-glutamic acid, 2,500 mg L-glutamine, 2,710 mg glycine, 10,415 mg L-histidine, 210 mg L-isoleucine, 315 mg L-leucine, 2,605 mg L-lysine hydrochloride, 210 mg L-methionine, 615 mg L-phenylalanine, 1,460 mg L-proline, 2,293 mg L-serine, 730 mg L-threonine, 415 mg L-tryptophan, 415 mg L-valine, 16,665 mg glucose, 2,085 mg fructose, 110,400 mg sucrose, 2,790 mg malic acid, 1,540 mg α-ketoglutaric acid, 250 mg succinic acid, and 230 mg fumaric acid. Bring up the solution to 1 l by the addition of distilled water. This stock solution contains

TABLE 10
Mitsuhashi's MGM-450 Medium

Components	mg/100 ml	Components	mg/100 ml
$NaH_2PO_4 \cdot 2H_2O$	95.8	L-Methionine	4.2
$NaHCO_3$	29.2	L-Phenylalanine	12.5
KCl	87.5	L-Proline	29.2
$CaCl_2$	83.3	DL-Serine	91.7
$MgCl_2 \cdot 6H_2O$	191.7	L-Threonine	14.6
$MgSO_4 \cdot 7H_2O$	233.3	L-Tryptophan	8.3
Glucose	333.3	L-Tyrosine	4.2
Fructose	41.7	L-Valine	8.3
Sucrose	2208.0	Thiamine·HCl	0.016
Malic acid	55.8	Riboflavin	0.016
α-Ketoglutaric acid	30.8	Pyridoxine·HCl	0.016
Succinic acid	5.0	Niacin	0.016
Fumaric acid	4.6	Calcium pantothenate	0.016
L-α-Alanine	26.3	Biotin	0.008
β-Alanine	16.7	Folic acid	0.016
L-Arginine·HCl	58.3	Isoinositol	0.016
L-Asparagine	29.3	*p*-Aminobenzoic acid	0.016
L-Cystine	2.1	Choline chloride	0.16
L-Glutamic acid	50.0	Inosine	20.0
L-Glutamine	50.0	Cytochrome c	10.0
Glycine	54.2	Polyvinylpyrrolidone K-90	50.0
L-Histidine	208.3	Fetuin	2.0
L-Isoleucine	4.2	Bovine plasma albumin	
L-Leucine	6.3	fraction V	1000.0
L-Lysine·HCl	52.1	Tryptose phosphate broth	300.0

Note: pH: 6.3. Osmolarity: 331 mOsm/kg. Additives: Fetal bovine serum 10% or fetal bovine serum 5% and *Antheraea pernyi* hemolymph 3%.

From Mitsuhashi, J. and Inoue, H., *Appl. Entomol. Zool.*, 23, 488–490, 1988. With permission.

five times concentrated amino acids, sugars, and organic acids (ASO), and it can be stored for several years at –20°C.

3. Stock Solutions of Vitamins (Vit-I and Vit-II)

The vitamin stock solutions are a thousand times concentrated. In order to prepare 100 ml of Vit-I and Vit-II, bring the pH of about 50 ml of distilled water to 10.0 with 0.1 *N* KOH. Dissolve in it 16 mg folic acid and 8 mg biotin. Adjust the pH to 7.0 with 1 *N* HCl. Bring up the solution to 100 ml by the addition of distilled water. This is Vit-I. Dissolve 16 mg riboflavin in 50 ml of distilled water preheated at 50°C. When the riboflavin dissolves completely, cool it to room temperature. Dissolve 16 mg calcium pantothenate, 16 mg pyridoxine hydrochloride, 16 mg *p*-aminobenzoic acid, 16 mg niacin, 16 mg isoinositol, and 160 mg choline chloride in the riboflavin solution. Add distilled water to the solution until the total volume becomes 100 ml. This is Vit-II. Both vitamin stock solutions can be stored for years at –20°C.

B. PREPARATION OF READY TO USE MEDIUM

In order to prepare 1 l of the medium, mix 100 ml ISM-A, 100 ml ISM-B, 200 ml ASO, 1.0 ml Vit-I, and 1.0 ml Vit-II. Add about 400 ml distilled water. Dissolve 10.0 g bovine plasma albumin fraction V, 20 mg fetuin, 100 mg cytochrome c (from horse heart), 200 mg

inosine, 500 mg polyvinylpyrrolidone K-90, and 3.0 g tryptose phosphate broth. Adjust the pH of the solution to 6.3 with concentrated KOH. Bring up the solution to 1 l by the addition of distilled water.

Other complex culture media may be prepared in the same manner.

V. PREPARATION OF MEDIUM ADDITIVES

A. PREPARATION OF INSECT HEMOLYMPH

Insect hemolymph by itself had been used frequently as a growth medium earlier, and also as a medium additive even at present. Insect hemolymph has phenol oxidase activity and it produces melanin when added to culture media. The resulting melanin is detrimental to cultured cells. It is, therefore, necessary to inactivate the phenol oxidase in the hemolymph. The following treatment is applied in most cases. Bleed insects and collect the oozed blood in a beaker or a centrifuge tube chilled on ice. An alternative receptacle is a beaker containing a small amount of liquid paraffin. Extract sufficient blood from several insects while cooling the receptacle on ice. The vessel containing liquid paraffin need not be cooled, because the blood sinks under the oil and does not contact the air. Immediately after obtaining sufficient blood, heat the blood to 60°C for 30 min in a water bath. Freeze the heat-treated blood at –20°C. The blood can be stored at this stage for many years. Thaw the frozen blood, and centrifuge at 4000 rpm for 15 min. Separate the supernatant. This is the insect hemolymph usable as a medium additive.

B. PREPARATION OF WATER-INSOLUBLE ADDITIVES

Some media are supplemented with some water-insoluble substances such as lipids. In order to incorporate these substances into media, the following procedures are usually applied. Dissolve chemicals in an appropriate organic solvent. Lipids such as fatty acids, glycerides, sterols, and phospholipids are usually dissolved in ethyl alcohol containing about 2% Tween 80 (as surfactants, lecithin, phosphatydylethanolamine, Pluronic F-68, and so on are also used instead of Tween 80). If chemicals are not soluble in ethyl alcohol, acetone may be used. Sterilize the solution by passing through a membrane filter which is tolerant to the organic solvent used. Ethyl alcohol has a strong germicidal property. Therefore, it is not necessary to sterilize the alcoholic solution. Add the solution to the sterilized culture medium under aseptic conditions. The amount of addition should be smaller than the maximum amount of solvent which does not cause any detrimental effects on cultured cells. The sensitivity of cells to organic solvents is different for different cell lines.

VI. STERILIZATION

For the sterilization of completed culture media, usually membrane filters are used. Membrane filters with a pore size smaller than 0.2 μm are used. In order to avoid clogging a filter with tiny particles present in the media, a series of prefiltration with larger pore size filters is recommended.

Some media such as MM medium can be sterilized by autoclaving (121°C 15 min), although this results in the formation of some precipitates, and cell growth-promoting ability is impaired to some extent.

VII. TEST FOR CONTAMINATION

The sterilized culture media should be left for several days at 25°C or at room temperature. The medium contaminated with bacteria or yeasts becomes turbid, and the one contaminated with fungal spores shows growth of hyphae. Sometimes, contamination of very slowly

growing microorganisms may occur. Detection of the contamination of such slowly growing organisms is very difficult.

VIII. STORAGE

The sterilized culture media can be stored for several months in a refrigerator. Media can be preserved below –20°C for several years. Some media such as MM or MGM-450 can be stored at room temperature for several months.

IX. MEDIUM IMPROVEMENT

A. SERUM-FREE MEDIA

Development of serum-free media is now a major trend of medium improvement. Serum-free media are preferable from the standpoint of medium cost as well as defining culture media. The crisis in FBS supply in the 1970s promoted development of serum-free media, and many serum-free media have been formulated. Some media were found to be adequate as serum-free media by simply eliminating the addition of sera. The MM medium was one of such media, and it supported the growth of various cell lines from different orders of insects. However, in most cases, substitutes for FBS were necessary to formulate serum-free media, because most insect cells depended on FBS for their growth. As a result of searching for such substances, the following were reported to support cell growth in lieu of FBS: bovine plasma (or serum) albumin,[22,23] pepton,[24] trypton,[25] lipid mixture,[26] liver digest,[27] cholesterol,[28] egg yolk,[29] etc.

Among these substitutes for FBS, lipids or lipid- containing substances are frequently used. Studies on lipid requirements by insect cells may give a clue to formulate chemically defined media.

Usually serum-free cultures of insect cells require some adaptive period. Most serum-free media kill insect cells when the serum-containing medium is completely replaced with serum-free medium at once. It is recommended to replace serum-containing medium with serum-free medium gradually. For this, the author has adopted the following method: when the density of cells increases enough for subculture, half of the cell suspension is discarded and an equal amount of serum-free medium is added. This procedure is repeated for more than ten passages. If the serum-free medium is not suitable for the cells, the growth rate of the cells decreases after five passages and all the cells would die before the tenth passage. If the serum-free medium is suitable for the cells, the cells grow with similar growth rate even after the tenth passage, when the concentration of substances derived from the serum is 2^{-10} of that of the original serum-containing medium. When this occurs, the medium can be replaced completely with serum-free medium. However, in the continuation of this procedure, sometimes the cells decline even after 20 passages. In this case, the reason of the decline can be hardly attributed directly to deficiency of substances carried over from serum-containing media. It appears reasonable to link the decline in activity of the cultured cells to the production of some factors, such as autocrine substances caused by the shortage of some substances in the medium. The occurrence of a very slow decline of cell multiplication points to the necessity of long-term evaluation of serum-free media.

B. CHEMICALLY DEFINED MEDIA

For chemically defined media, at present only one medium, CDM formulated by Wilkie et al. (Table 5) is available for culturing established cell lines. Notable characteristics of this medium

are incorporation of trace metals, polyamines, and lipids such as fatty acids, triglycerides, sterols, and phospholipids. This medium is difficult to prepare, because some components are hardly soluble in water. Some industries once sold this medium, but currently no industries supply this medium. Chemically defined media without protein or peptides are preferable. Proteins in a medium may result in unstable amino acid composition of the medium, because of the degradation of the proteins. Protein-free media will therefore be preferable for biochemical studies on insect cells, as well as for the use of insect cell cultures for the production of foreign proteins by means of recombinant baculoviruses.

A strategy to develop such a medium is to formulate a chemically defined basic medium which can support cell growth when supplemented with FBS, and then to find chemically defined substitutes for the FBS. The development of such basic media is not difficult. However, the construction of FBS substitutes will be difficult. Many investigators have tried to substitute FBS with other substances, but unfortunately the substitutes found to be effective are all undefined (Table 11). Among them, Maiorella's supplements contain only a small amount of unknown substances (Tables 9 and 11). It may be possible to develop a chemically defined medium from this medium. Table 11 suggests the importance of lipids, because all the substitutes contain lipids.

Vaughn and Louloudes[35] have reviewed the lipid requirement of cultured insect cells. From their review, it became evident that growth of many insect cell lines is promoted by incorporation of a mixture of lipids. Among lipids, some polyunsaturated fatty acids are assumed to be growth stimulatory, based on the lipid analyses of cultured insect cells, as well as the essentiality of fatty acids for growth of intact insects. However, the effects of individual fatty acids on the growth of the cultured insect cells are still not evident.

On the other hand, it is also true that some insect cells, such as a cabbage armyworm cell line, NIAS-MB-32, or a fleshfly cell line, NIH-SaPe-4, can grow continuously in a lipid-free medium such as MTCM-1103 (Table 12). In the lipid-free medium, the cell growth was somewhat slower compared with medium containing FBS.[7] Table 13 shows fatty acid analysis of cells cultured in the lipid-free medium. Insect cells could synthesize most of saturated fatty acids, such as myristic acid, palmitic acids, stearic acid, and some unsaturated fatty acids, such as palmitoleic acid and oleic acid, even when they were cultured in the medium free of lipids.[36] They cannot, however, synthesize polyunsaturated fatty acids such as linoleic acid and linolenic acid. Therefore, addition of polyunsaturated fatty acids to the medium may improve the cell growth. Cod liver oil used in the Maiorella et al. medium has been known to contain polyunsaturated fatty acids such as octadecatetraenoic acid, eicosapentaenoic acid, docosahexaenoic acid, and so on. These fatty acids may be important in this medium. Egg yolk, known to contain various lipids, has also been used by Röder[29] and Léry and Fedière.[34] Tables 14 and 15 show lipid composition of egg yolk. Egg yolk contains various lipids such as fatty acids, phospholipids, steroids, carotenoids, and vitamins. Among the fatty acids are many polyunsaturated fatty acids.

The effect of a mixture of lipids contained in FBS should be also examined. Published data on lipid analyses of FBS are fragmental. In vertebrate cell cultures, various growth factors are used in chemically defined media. In insect cell cultures, however, no growth factors proper to insect cells nor vertebrate growth factors effective to insect cells have been known except insulin for *Drosophila* cells.[37] Insect hemolymph, FBS, and lactalbumin hydrolysate are known to contain growth-promoting substances. For a long time, several investigators have been trying to isolate growth-promoting substances from these materials, but no one has succeeded as yet. The difficulty seems to come from attenuation of the activity upon fractionation. If a growth factor effective for insect cells were found in near future, it would facilitate the development of chemically defined media.

TABLE 11
Substitutes for Fetal Bovine Serum in Serum-Free Media with Chemically Defined Base

Substances	Ref.
Lactalbumin hydrolysate, yeastolate, bactotryptose	25
Trace metal, fatty acids, sterols, polyamines, phospholipids	8
Chicken egg yolk	29
Tryptosephosphate broth	30
Lactalbumin hydrolysate, yeastolate, polyamine, sterols, trace metals, nucleic acid precursors	31
Ultroser G (LKB)	32
Nuserum (Collab. Res.)	32
Fetal bovine serum albumin (K.C. Biol.)	32
Cod liver oil, sterols, vitamin E, tryptosephosphate broth, pluronic polyol F-68, yeast ultrafiltrate	20
CPSR-1, -3 (Sigma)	33
Yeastolate, polyamines, sterols, nucleic acid precursor, liver digest, tryptose	27
Polyamines, chicken egg yolk	34

TABLE 12
Mitsuhashi's MTCM-1103 Medium

Components	mg/100 ml	Components	mg/100 ml
NaCl	700.0	Thiamine·HCl	0.016
NaH_2PO_4	20.0	Riboflavin	0.016
$NaHCO_3$	12.0	Calcium pantothenate	0.016
KCl	20.0	Pyridoxine·HCl	0.016
$MgCl_2 \cdot 6H_2O$	10.0	*p*-Aminobenzoic acid	0.016
$CaCl_2 \cdot 2H_2O$	20.0	Folic acid	0.016
Glucose	500.0	Niacin	0.016
Inosine	20.0	Inositol	0.016
Lactalbumin hydrolysate	1500.0	Biotin	0.008
Choline chloride	0.16		

Note: pH: 6.5. Osmolarity: 347 mOsm/kg. Used as a serum-free medium.

From Mitsuhashi, J., *Appl. Entomol. Zool.*, 17, 575–581, 1982. With permission.

C. LOW-COST MEDIA

For low-cost media, serum-free cultures are a prerequisite. Currently most insect cell lines are cultured in media supplemented with FBS. FBS was first used for insect tissue culture by Martignoni and Scallion in 1961.[38] A few years later, the growth-promoting ability of FBS for insect cells was ascertained by Mitsuhashi and Maramorosch.[6] Since then, most insect cell culturists have employed FBS for their culture media. However, FBS is very expensive, and therefore cannot be used for large-scale cultures at high concentrations. Fortunately, many serum-free culture media have been developed recently.

For large-scale cultures, media should be prepared easily and should consist of low-cost ingredients. A serum-free medium consisting of many ingredients is not practical, because the preparation would be time consuming. Another preferable property of media for large-scale culture is tolerance for autoclaving. Usually culture media are sterilized by passing through a sterilizing filter. Often, the filter gets clogged with particles in solutions. A serial prefiltration may be needed to avoid this sort of trouble. These procedures are also time consuming, and become a very big problem when large quantities of media are sterilized. If the medium is autoclavable, such troubles can be eliminated.