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DEVELOPMENT OF A MEDIUM FOR THE CULTURE OF AGALLIAN LEAFHOPPER CELLS

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The study of certain plant viruses that multiply in the leafhopper vector has been facilitated by the development of a tissue culture technique for cells of the vector. The technique probably will advance the study not only of such plant viruses but of mycoplasma- and rickettsia-like pathogens transmitted by leafhoppers to plants. Research in this field has been retarded because of the limitations of the insect tissue culture medium and by the lack of a simple procedure for determining optimal physical and nutritional conditions for growth. In this study, Agallia constricta cells, one of the few leafhopper cell lines that has been successfully established, were used to determine the optimal conditions for their growth and to improve the medium initially used for their establishment and continuous subculture.

A procedure was developed for the accurate, quick, and simple indexing of growth by counting nuclei under the points of a reticule in the eyepiece of an inverted phase contrast microscope at a magnification of 500x. In certain experiments, it was necessary to obtain a measure of the area of the nuclei being counted, and to correct nuclear counts according to the relative areas of nuclei being compared. The counting technique permitted handling large numbers of treatments simultaneously because of the small number of replicates necessary and the small amount of time required to make the counts. On undisturbed cell monolayers, the technique made more information

available than did other standard counting procedures, such as counts by hemocytometer or electronic cell counter.

Differences were observed in the suitability of batches of fetal bovine serum, lactalbumin hydrolysate and histidine as components in the insect tissue culture medium. These differences were demonstrated in samples from different commercial suppliers and in samples from a single supplier.

Optimal concentrations of salts, dextrose, lactalbumin hydrolysate, yeast hydrolysate, fetal bovine serum, histidine and antibiotics were determined. Fungizone (amphotericin B) at 50 or 100 mg per liter caused harmful effects. Optimal growth of A. constricta cells occurred at about pH 6.43, a pH that was also optimal for Agalliopsis novella cells. Optimal growth for Aceratagallia sanguinolenta was at pH 6.3. Optimal osmotic pressure varied in different media from 310 to about 360 mOsm, and optimal temperature was about 28°C. Short term storage of cells at 13°C with changes of medium every 3 to 4 months was very convenient. A. constricta cells were recovered after 21 months storage at this temperature.

Doubling time of A. constricta cells was reduced from about 74 hours in medium O to about 60 hours in medium A, about 45 hours in medium B. The doubling time in medium D between 24 and 48 hours after subculture was about 23 hours. Insect cells subcultured in the improved medium had a better appearance as well as a shorter doubling time. The susceptibility of cells to wound tumor virus or to potato yellow dwarf virus was greater when they were grown in the improved medium.

Papain at 0.01 mg/ml was an excellent enzyme for the detachment of the cells at time of subculture, perhaps because its optimal activity at pH 6.4 is the same as the optimal pH for growth of the cells.

The development of the counting technique with the phase contrast microscope and the formulation of a medium that supports growth at about twice the rate achieved in the only medium in which establishment of leafhopper cell lines has been achieved, may prove to be advantageous for the establishment of other leafhopper cell lines and possibly of cells of other insect vectors of plant pathogenic microorganisms.

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INTRODUCTION

Since the establishment of cells of Anthereae eucalypti Scott, in continuous culture by Grace (1962), much work has been done in establishing or in attempting to establish other insect cell lines. Much work has also been carried out in using primary cultures and established cell lines in various ways, among them in the study of plant pathogens transmitted by insects. Primary cultures of insect vectors have been used in the study of plant pathogens (Vago and Flandre, 1963; Mitsuhashi, 1965a, b; Chiu and Black, 1966; Peters and Black, 1970, 1971; Shikata et al, 1970; Yamada et al, 1970), but most progress has been achieved after the successful establishment of continuous cell lines of Agallia constricta Van Duzee and Aceratagallia sanguinolenta (Provancher) of several number designations by Chiu and Black (1967). With these cell lines it has been possible to carry out precise investigations of many problems concerning those plant viruses that multiply in vectors from which the cells were derived, because of the sensitivity of the technique and the short intervals required to obtain the results (Chiu and Black, 1967, 1969; Gamez and Chiu, 1968; Black, 1969; Liu, 1969; Liu and Black, 1969; Chiu et al, 1970; Hsu, 1971; Hsu and Black, 1970, 1971, 1972, 1973a, b, c; Kimura and Black, 1971, 1972a, b; Reddy and Black, 1972, 1973; Liu et al, 1973).

These cell lines were obtained using a modification of Schneider's (1964) Drosophila tissue culture medium (Chiu and Black, 1967). The leafhopper medium of Chiu and Black (1967) was also used in the establishment of cell lines of Colladonus montanus (Van Duzee) (Richardson and Jensen,

1971) and in the establishment of cell lines of Agalliopsis novella (Say) by Windsor (1972).

The established leafhopper cell lines have made it possible to obtain the large number of cells required to carry on studies of optimal physical and nutritional conditions for the growth of the cells. Lack of knowledge about these conditions has been the limiting factor in the development of insect tissue culture techniques (Vaughn, 1967; Brooks and Kurtti, 1971).

The preparation of most insect tissue culture media has been based on analyses of insect hemolymph (Wyatt, 1956, 1961; Wyatt et al, 1956; Grace, 1962; Vago and Flandre, 1963; Schneider, 1964; Plantevin, 1967). This has not been a very fruitful approach possibly because of the differences observed in the composition of hemolymph between individual insects and between different developmental stages (Florkin and Jeaunoux, 1964; Wigglesworth, 1965; Schwemmler et al, 1971; Demal and Leloup, 1972). In the case of leafhopper tissue culture, where a complete analysis of leafhopper hemolymph has not been performed, the media used has been developed through trial and error (Vago and Flandre, 1963; Hirumi and Maramorosch, 1964; Mitsuhashi and Maramorosch, 1964; Mitsuhashi, 1965a, 1969; Chiu et al, 1966; Chiu and Black, 1967; Shikata et al, 1970). This may explain the failures of most investigations to establish cell lines of the vectors and the slow development of the field.

The procedure most widely used for the determination of optimal conditions for growth of cells has been the measurement of the increase in the number or volume of the cells (Parker, 1961; Paul, 1970). Several procedures have been used to get this information, most of which involve the harvesting of the cells from the culture substrate, followed by dilutions and direct enumeration of cell numbers with a hemocytometer or an electronic cell

counter. Some other methods measure the packed cell volume, dry or wet weight, or some cellular constituent such as protein or deoxyribonucleic acid (Parker, 1961; Paul, 1970). Enumeration of the total number of cells in each microscopic field has been used (Jamieson et al, 1969), and the enumeration of the cells in ten squares on a diagonal line of a grid ocular micrometer in eight different microscopic fields was used by Mitsuhashi et al (1970). Recently, microphotographic methods have been employed (Castor, 1971; Froeze, 1971; Gerschenson et al, 1972). Schlegel and Black (1969) studied the optimal temperature for the growth of Agallia constricta cells using a hemocytometer. Martinez-Lopez and Black (1971) used the Coulter Cell Counter to investigate the optimal temperature, to determine the optimal concentration of fetal bovine serum in the leafhopper medium of Chiu and Black (1967) and to study the effect of infection by wound tumor virus on the cells.

In this study an attempt has been made to determine the optimal conditions for the growth of a cell line (AC20) derived from the leafhopper A. constricta. Some of the difficulties encountered with the use of the Coulter Cell Counter were overcome by the development of an easier technique to evaluate the growth of the cells under different conditions. In this technique cell nuclei were enumerated in situ with the use of a reticule in the eyepiece of an inverted phase contrast microscope. The number of cell nuclei that coincided with certain points in the reticule were counted and the count was used as an index of the number of cells in a given flask. This index was used in the determination of the optimal concentration of each one of the medium components and in the formulation of an improved tissue culture medium. The optimal pH, osmotic pressure and temperature for growth and for short term storage of the cells, as well as the effect of the optimal conditions on the susceptibility of the cells to virus infection were studied.

MATERIALS AND METHODS

A. Tissue Cultures of Vector Cells

Cell lines of the leafhoppers Agallia constricta Van Duzee and Ceratagallia sanguinolenta (Provancher) established by Chiu and Black (1967) and Agalliopsis novella (Say) established by Windsor (1972) were used throughout the experiments. The abbreviations used for the cell lines of these three species were AC, AS and AN, respectively. Each cell line was also given a number which signified the numerical order of its isolation from the species. AC20 was the best of the AC cell lines isolated and most of the work was done with it.

B. Methods of Tissue Culture

Stock and experimental cultures were grown in 25 cm^2 disposable plastic flasks (Falcon Plastics, Los Angeles, California) and incubated at a temperature about 28°C, unless otherwise stated. Subcultures were usually made every 4 to 5 days. In this study the original medium of Chiu and Black (1967) is designated medium O (Table 1). This study was begun with the medium of Martinez-Lopez and Black (1971), i.e. medium A (Table 1). Later as additional improvements were made in the medium the various media formulations were identified by still other letters. A culture of AC20 cells was kept in medium A only, as a control.

When virus infectivity studies were involved, cell monolayers were grown on glass coverslips of 15 mm diameter (Propper Mfg. Co., Inc., New York) previously washed in acetone (reagent grade) in an ultrasonicator. Usually glass petri dishes (Bellco Glass Inc., Vineland, New Jersey) were used to

Table 1

COMPOSITION OF DIFFERENT MEDIA USED TO CULTURE AC20 CELLS*

	Medium					
	O	A	B	B_1	C	D
$CaCl_2.2H_2O$	400	400	400	400	360	360
$MgSO_4.7H_2O$	1,853	1,853	1,853	1,300	1,170	1,200
KH_2PO_4	300	300	300	300	270	270
KCl	800	800	800	1,600	1,600	1,600
NaCl	1,053	1,053	1,053	1,000	1,000	1,000
$NaHCO_3$	353	353	353	400	400	900
Dextrose	4,000	4,000	4,000	6,000	5,400	9,000
Lactalbumin-hydrolysate	6,500	6,500	6,500	10,000	10,000	10,000
Yeast hydrolysate	5,000	5,000	5,000	8,000	8,000	8,000
Fetal bovine serum	200 ml	100 ml	100 ml	100 ml	100 ml	100 ml
L-Histidine HCl (monohydrate)	0	0	4,720	4,720	4,720	5,093
L-Histidine (free base)	0	0	4,270	4,270	4,270	3,212
Penicillin G	100,000 units	100,000 units	100,000 units	100,000 units	100,000 units	100,000 units
Streptomycin	100	100	100	100	100	100
Neomycin	50	50	50	50	50	50
Fungizone	25	25	25	25	25	0
pH	ca 7.1	ca 7.1	6.43	6.43	6.43	6.43
Osmotic pressure	250 ± 5 mOsm	205 ± 5 mOsm	273 ± 5 mOsm	344 ± 5 mOsm	310 ± 5 mOsm	360 ± 5 mOsm

*Numbers in table refer to mg/liter unless specified otherwise.

contain the coverslip cultures, 2 to each dish, and these were sealed with nontoxic silicone lubricant (High Vacuum Grease, Dow Corning Corporation, Midland, Michigan).

In most experiments the cells were dissociated and detached from the flasks by the trypsinization procedure described by Chiu and Black (1967). After removing growth medium from the flask, the cells were washed once with 2 ml of Rinaldini's solution (1959) at pH 7.5. This wash solution was removed and replaced with 2 ml of Rinaldini's solution containing 0.05 per cent trypsin (Trypsin 1:250 DIFCO, Detroit, Michigan). After 5-6 minutes incubation at about 25°C, the tryptic action was ended by dilution with 2 ml of growth medium. The cells were removed from the flask by flushing them free with a disposable Pasteur pipette and were transferred to a tube which was capped and centrifuged at an average force of 63 g for 2 minutes in an International Clinical Centrifuge (Model CL). The supernatant above the sedimented cells was discarded and replaced with a volume of fresh medium that depended on the number of new flasks to be seeded. Each flask was usually seeded with 4 ml and a sufficient volume of suspension was prepared to permit recovery of the last aliquot of 4 ml exactly by leaving a small residual excess volume.

Some modifications were made in this procedure and other concentrations of trypsin and times for digestion were tested. The possibility of using papain for the enzymatic detachment of the cells was studied and after it was found more suitable than trypsin for the AC20 cells, this enzyme was used in all subsequent experiments. The development of the papain procedure is described in "Experiments and Results."

Special care was taken to obtain an even distribution of seeded cells on the bottom surface of the flask. A homogeneous suspension of the cells

was obtained by using a mixer (Vortex-Genie Model K-550G) to stir the cells in the centrifuge tube at least 5 times at its maximum setting for 2-3 seconds each time. The degree of homogeneity was judged by the subsequent distribution of cells on the bottom surface of the flask to which the cells were transferred. After adding a cell suspension to the flask it was moved constantly until it had been put on a leveled rack in the incubator. This constant movement was designed to keep the longitudinal axis horizontal while at the same time rotating the flask back and forth about the axis through a short arc.

In each one of the experiments in which different media were compared, stock cultures were harvested using the control medium for quenching the activity of the enzyme. After the cells had been transferred to a centrifuge tube a homogeneous suspension was obtained with the aid of the Vortex mixer, and equal aliquots, assumed to contain the same number of cells, were distributed in as many centrifuge tubes as necessary, according to the number of flasks to be used in the test. After the cells were centrifuged and the supernatant removed, they were suspended in the medium to be tested, seeded in 4 ml of the suspension and left to grow for 4 days without change of medium. In different experiments the number of cells added to each flask varied from 1.5×10^6 to 2.5×10^6; the number present 4 days later after satisfactory growth varied from 6×10^6 to 8×10^6.

Some cultures were kept at 13°C for various intervals (Hotpack Refrigerated Incubator Model 352700) to study storage at this temperature with widely spaced changes of growth medium (Liu, 1969). When it was desired to hold cultures for a few days or weeks before using them they were also stored at this temperature. For long storage, cultures were stored at -196°C according to the procedure described by Hsu and Black (1969).

C. Preparation of Media

The composition of the various media are given in Table 1. With the exceptions noted below, concentrated solutions of each component were prepared separately in suitable small volumes, stored frozen at -25°C and thawed just before being used to make media. Lactalbumin hydrolysate was prepared fresh each time by adding distilled water to a weighed amount of the powder, warming the mixture to 50°C until solution was completed, cooling the solution to room temperature and adjusting to the final volume desired. Some samples of lactalbumin hydrolysate dissolved completely without warming to 50°C.

The toxicity observed with many mammalian sera is eliminated by heat treatment (Parker, 1961). Bottles of fetal bovine serum were thawed by putting them in tap water at room temperature. Before all the ice was melted they were placed in an ice bath for a short time as a matter of convenience before they were heated quickly in boiling water from 0 to 56°C with stirring. They were then transferred to a water bath and kept at 56°C for 30 minutes before being cooled.

Starting with component #1 each one of the component solutions was added in succession to the volume of distilled water required to give the correct final volume. Stirring was continuous to avoid precipitation of some of the salts. The mixture was adjusted to the desired pH, if necessary, and then was sterilized by filtration in a previously autoclaved 142 mm Millipore filter holder in which 0.8, 0.45 and 0.22 µm filters had been arranged serially with progressively finer sizes. On top of the 0.8 µm filter a prefilter was used, and the filters were separated from one another with dacron separators. These filters did not have the small amount of wetting agent sometimes included in them (Millipore Corp., Bedford, Massachusetts).

The preparation of medium D, the final formulation, departed slightly from this general procedure and is described in detail on page 83.

D. Determinations of pH

pH was measured at room temperature (about 25°C) with a pH-101 Metrohm pH meter (Brinkmann Instruments, Westbury, New York) equipped with an EA125X combined electrode, and calibrated with standard buffers from the National Bureau of Standards, U.S. Department of Commerce. The buffers were dissolved in glass distilled water that had a specific conductance at 25°C of 1.25 micromhos measured in a conductivity bridge Model RC16B2 equipped with conductivity cell CEL-A01 K-0.100/CM.

E. Osmotic Pressure Determinations

Experiments were performed to measure the growth of the insect cells under a range of osmotic pressures of the medium. These were measured in a Fiske Osmometer (Fiske Associates, Inc., Bethel, Connecticut).

F. Virus Inocula

1. Wound tumor virus inoculum

When wound tumor virus (WTV) was used to inoculate insect tissue cultures (Black, 1969), inoculum of the vectorial virus VI72, purified and stored at -80°C, was provided by Dr. D. V. R. Reddy. The virus isolate (VI72) is fully transmissible by the vector A. constricta. The properties of this particular batch of inoculum have been defined by Reddy and Black (1973).

2. Potato yellow dwarf virus inocula

When infectivity studies were made with constricta yellow dwarf virus (CYDV) or sanguinolenta yellow dwarf virus (SYDV) (Black, 1970), purified inoculum of vectorial virus isolates of these two viruses that had been stored at -80°C was provided by Dr. H. T. Hsu.

G. Measurement of Cell Growth

1. Cell counting procedures

a. Coulter Counter

Cell suspensions were obtained by the same procedure used for harvesting cells for subcultures. The procedure described by Martinez-Lopez (1971) was used routinely. After suspending the cells in fresh medium and stirring with the Vortex-Genie mixer, 2 ml samples were taken and each was diluted in 12 ml of 0.9 per cent NaCl previously filtered through a 0.45 µm Millipore filter to remove large particles in the diluent solution that might interfere with counts. The sample was again stirred with the Vortex-Genie mixer and maintained at 0^{o}C until the counts were made.

Counts were made with the Coulter Counter, Model B (Coulter Electronics, Inc., Hialeah, Florida), under the following conditions:

aperture tube	100 µm
manometer size	500 microliters
1/amplification	setting 1
1/aperture current	" 2
matching switch	" 64H
gain control	" 52
lower threshold control	" 10
upper threshold control	" 100

These conditions were chosen after several combinations were tested and compared with direct counts in a hemocytometer. At least three counts were made on each sample. The average of these three counts was corrected using the correction chart for the 100 µm aperture. It was not necessary to make any correction for background counts because the diluent gave less than 10

counts per 500 microliters. The counts represented measures of absolute numbers of cells and from the dilution used during sampling could be calculated the number of cells harvested per flask.

b. Enumeration of cell nuclei with the phase contrast microscope

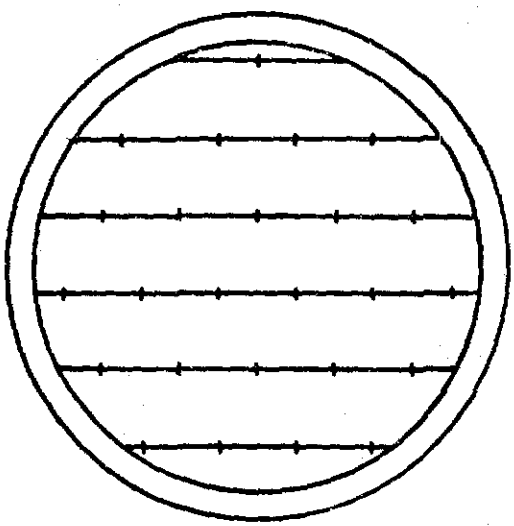
Cell nuclei were enumerated in an inverted phase contrast Zeiss microscope equipped with a Ph2 Plan 40/0.60 objective lens, a body magnification of 1.25x and a Kpl 10x eyepiece having a reticule with 25 points distributed systematically over the whole field and connected by straight lines to facilitate orientation during counting (Fig. 1). The total magnification used during counting was 500x. Experimental flasks to be studied were held in the mechanical stage of the microscope. Cell nuclei that coincided with any one of the 25 points in the reticule were counted in each of 5, 10, 20 or 40 microscopic fields per flask. The position of the fields (Fig. 2) were selected by displacement of the stage in the two coordinate directions to fixed values in the longitudinal and transverse scales.

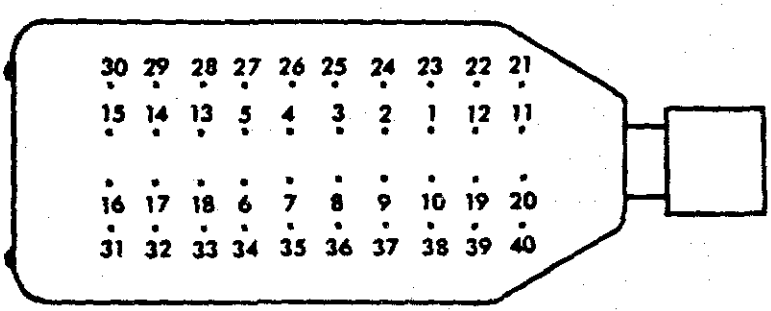
Counts were made in each one of the experimental flasks 24, 48, 72 and 96 hours after subculture, and the average number of counts over this 4-day period was used to determine the difference in the rate of growth between the media tested. The highest average count was considered as 100% and each one of the other averages was expressed as a percentage of the best.

In this investigation it has been assumed that measurements of the number of nuclei would provide a satisfactory measure of growth. Because of the advantages of the above technique most experiments on growth of the insect cell monolayers were made by direct enumeration of nuclei under the phase contrast microscope.

Fig. 1. Diagram of the reticule with 25 points distributed systematically over the microscopic field and connected by straight lines to facilitate orientation during counting. (x4.87)

Fig. 2. Outline of a flask with numbered points indicating the location of microscope fields in which nuclei were enumerated. (x1.0)




30 29 28 27 26 25 24 23 22 21
15 14 13 5 4 3 2 1 12 11
16 17 18 6 7 8 9 10 19 20
31 32 33 34 35 36 37 38 39 40

c. Doubling time

In some of the work it appeared that counts of nuclei made by the method described above when applied to monolayers with crowded cells, may have underestimated the number of nuclei relative to the number in uncrowded monolayers. It was hypothesized that this might occur because of the average area of the nuclei being less in crowded monolayers. Because the chances of any nucleus coinciding with a reticule point must be proportional to its area a second method of counting was examined in which counts of nuclei were corrected by measures of nuclear area when comparisons were made between sets with nuclei that might have a different average area. The area of a nucleus was taken as the area within its circumference when its equator was in focus. Areas were calculated by using a micrometer scale in a 12.5x ocular lens to determine the diameters of those nuclei coinciding with the reticule points. The circumference of the equatorial plane of some nuclei was somewhat elliptical instead of circular; the area of such nuclei was taken as the area of a circle with a diameter equal to the lesser diameter of the ellipse. Any small error so introduced was ignored in the present study.

H. Infectivity Tests with Insect Cell Monolayers

The inoculation procedures described by Kimura and Black (1971) for WTV and Hsu and Black (1973b) for SYDV and CYDV were followed. Cells were incubated for 40 to 48 hours at about 28°C before cells were fixed. Virus infection of insect cell monolayers was detected by the direct method of fluorescent antibody staining developed by Coons et al (1942) and used for leafhopper-borne virus studies by Nagaraj et al (1961), Chiu et al (1966), Chiu and Black (1967, 1969), Liu (1969), Kimura and Black (1971, 1972a, b), Hsu (1971), Hsu and Black (1970, 1971, 1972, 1973a, b, c) and Reddy and

Black (1972, 1973). Examination for infected cells was made with a Leitz Ortholux fluorescence microscope using either transmitted or incident light. Infected cells were counted under a 40x objective lens (Leitz NP 1 40/0.65) and an 8x ocular lens.

Comparable index values for the proportion of infected cells were determined by using the procedure described by Kimura and Black (1972b, p. 559). In this procedure the total number of infected cells in each one of the central 13 microscopic fields in the vertical and horizontal diametral zones were counted. At the same time the total number of cells in the central square of a reticule, a square that corresponded to 1/50 of the total area in the microscopic field, was counted and used to determine each index value. It was necessary to determine this index because of the differences in the number of cells in each of the monolayers depending on the medium being used for growing them.

EXPERIMENTS AND RESULTS

Evaluation of Different Factors Involved in the Growth of AC20 Cell Monolayers

A. Cell Growth Measurements

The Coulter Cell Counter was used to measure the growth of insect cell monolayers as described in "Materials and Methods," but some difficulties were encountered with this method. In early experiments on the effect of the pH of the medium on cell growth, the tendency of the cells to aggregate after treatment with trypsin interfered with the counting procedure in the electronic cell counter. Some gross errors in the cell counts led to the development of the counting technique with the phase contrast microscope described in "Materials and Methods." It was not seriously affected by the aggregates and has a number of other advantages. It was used in all measurements unless otherwise specified.

As a result of extensive observations of the cells under the phase contrast microscope under a variety of conditions under which the nuclei were being counted judgments have been formed as to the appearance of healthy and unhealthy cells. Cells that grew more rapidly had few or no small spherical globules that appeared yellowish; in contrast there were large numbers of such globules in cells that were growing slowly or not at all. In the former case there were many pseudopodia with many extended branches and fine tips; in the latter the pseudopodia were few, short, unbranched and blunt. In the former case the periphery of the nucleus was clearly defined, in the latter it was sometimes unclear and diffuse. In the former case the protoplasm was much more dense than in the latter. In the former case there was little or

no debris whereas in the latter case there was often a great deal. When growth is rapid, cells in mitosis are common. Cells that showed the various characteristics associated with rapid growth as described above were said to look better or healthy in comparison with those that appeared poor or unhealthy by showing the opposite characteristics. When growth is slow because of low temperature (e.g. 13°C) the appearance of the cells may be healthy but cells in mitosis may be rare.

1. Evaluation of the number of counts required per experimental unit

The number of counts required for an accurate determination of the index of cell growth with the phase contrast microscope was determined experimentally. The number of cell nuclei coinciding with the points in the reticule were counted in 5, 10, 20 and 40 microscopic fields in each one of four flasks, two of which had an average of 3.2 cell nuclei, and two 6.0 nuclei, coinciding with the points. Table 2 illustrates the results obtained from the four flasks tested. When the variance was analyzed, no significant differences were observed between the different number of counts (Table 3). Obviously one of the main factors making this possible was the uniform distribution of the cells on the monolayer. When the 95% confidence limit of the mean was determined by using the equation $L = \frac{1.96\sigma}{\sqrt{n}}$ (Snedecor and Cochran, 1967), the limit became shorter when the number of counts was increased (Table 4). The 95% confidence limit as a percentage of the mean ($L_p = \frac{1.96\sigma/\mu}{\sqrt{n}}$ showed that it was similar for both low and high cell densities (Table 4).

It appeared that the simple enumeration of nuclei in 10 microscopic fields usually provided satisfactory information on a single flask and it took less than 5 minutes to complete. Counts of 20 fields instead of 10

Table 2

AC20 NUCLEI COUNTED PER MICROSCOPIC FIELD

Number of microscopic fields counted	Flask	Flasks with low cell density				Flasks with high cell density			
		Mean	Standard deviation of the observations	Standard error of the mean	Coefficient of variation	Mean	Standard deviation of the observations	Standard error of the mean	Coefficient of variation
5	(1)	3.4	1.5	0.67	0.44	6.0	2.0	0.89	0.33
	(2)	3.6	2.0	0.89	0.56	6.2	2.6	1.16	0.42
10	(1)	3.4	1.4	0.44	0.41	6.3	1.8	0.57	0.29
	(2)	3.1	1.1	0.35	0.35	6.4	2.7	0.85	0.42
20	(1)	3.2	1.3	0.29	0.39	5.9	2.2	0.49	0.37
	(2)	3.2	1.2	0.27	0.38	5.5	2.0	0.44	0.36
40	(1)	3.1	1.4	0.22	0.44	5.8	2.1	0.33	0.37
	(2)	3.2	1.2	0.19	0.38	6.1	2.0	0.32	0.33
Average		3.2	1.3		0.40	6.0	2.2		0.36

Table 3

ANALYSIS OF VARIANCE FOR DATA OF TABLE 2

Source of variation	Degrees of freedom	Mean square	
		Low cell density	High cell density
Between treatments	3	.52	1.66
Replication/treatment	4	.61	1.0
Within flask	142	1.78	4.64
Total	149		
		$F_{cal} = \frac{0.52}{0.61} = 0.85^*$	$F_{cal} = \frac{1.66}{1.00} = 1.66^*$

*With a confidence probability of 95% F_{cal} must be greater than 6.5914 to have significant differences.

Table 4

EFFECT OF COUNTING NUCLEI IN VARIOUS NUMBERS OF MICROSCOPIC FIELDS PER FLASK ON THE 95% CONFIDENCE LIMIT OF THE MEAN (L) AND 95% CONFIDENCE LIMIT AS A PERCENTAGE OF THE MEAN (L_p)

Number (n) of microscopic fields counted	Low cell density $L = \frac{1.96\sigma}{\sqrt{n}}$*	Low cell density $L_p = \frac{1.96\sigma/\mu}{\sqrt{n}}$**	High cell density $L = \frac{1.96\sigma}{\sqrt{n}}$*	High cell density $L_p = \frac{1.96\sigma/\mu}{\sqrt{n}}$**
5	1.20***	0.36	1.87	0.31
10	0.85	0.25	1.32	0.22
20	0.60	0.18	0.93	0.15
40	0.42	0.13	0.66	0.11

* σ = standard deviation

** σ/μ = coefficient of variation

*** Each of these calculated numbers is a measure of the precision of the mean.

increased the sensitivity of the technique so that smaller differences between treatments could be determined.

2. Sensitivity of the counting technique and comparisons between members of pairs of flasks

The sensitivity of the counting technique with the phase contrast microscope was tested using it to compare 8 lots of media differing from one another only in that each contained a different commercial batch of lactalbumin hydrolysate as one of the components. Comparisons were made between each two flasks containing the same lot of medium and comparisons were also made between media containing different batches of lactalbumin hydrolysate (Tables 5 and 6). When compared statistically by the F test no significant differences were observed between any member of a pair having the same batch of lactalbumin hydrolysate, but significant differences were demonstrated between a number of different batches of lactalbumin hydrolysate (Table 7). Batch B appeared to be the best, but no significant difference was observed when it was compared with batch F. Some batches appeared to be suitable and others unsuitable as a component in the culture medium for Agallian cells even when some of the batches came from the same company. Batch B was selected for the formulation of media B, C, and D (Table 1).

B. Optimal Concentration of Fetal Bovine Serum

Martinez-Lopez and Black (1971) determined that a concentration of 10% fetal bovine serum (FBS) was as good or even better than the 20% concentration used previously in medium O (Chiu and Black, 1967). In order to determine how important this 10% concentration was for the growth of the cells, medium B was prepared with 5, 7.5 and 10% FBS and AC20 cells were grown in

Table 5

COUNTS OF NUCLEI IN CELL MONOLAYERS GROWN IN MEDIA CONTAINING DIFFERENT COMMERCIAL BATCHES (A TO H) OF LACTALBUMIN HYDROLYSATE

Lactalbumin hydrolysate batch	Flask	Mean number nuclei	Variance	Standard deviation	Coefficient of variation
A	(1)	1.5	1.0	1.0	0.66*
	(2)	1.5	1.5	1.2	0.80*
B	(1)	5.9	4.3	2.0	0.33
	(2)	5.7	2.3	1.5	0.26
C	(1)	3.0	1.1	1.0	0.33
	(2)	3.1	1.0	1.0	0.32
D	(1)	2.5	1.0	1.0	0.40
	(2)	2.9	1.2	1.1	0.37
E	(1)	2.8	1.3	1.1	0.39
	(2)	2.6	1.2	1.1	0.42
F	(1)	5.3	2.3	1.5	0.28
	(2)	5.0	2.0	1.4	0.28
G	(1)	3.4	1.2	1.1	0.32
	(2)	3.3	1.0	1.0	0.30
H	(1)	3.5	1.8	1.3	0.37
	(2)	3.0	0.7	0.8	0.26

*This high coefficient of variation was associated with a very poor condition of the cell monolayer.

Table 6

ANALYSIS OF VARIANCE OF DATA IN TABLE 5

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatment	15	273	18.2
Error	144	223	1.54
Total	159	496	

$F_{cal} = \frac{18.2}{1.54} = 11.8$ (For a confidence probability of 95% F is 1.75; the differences between treatments are therefore highly significant.)

Table 7

F_{cal} VALUES FOR COMPARISONS POSSIBLE IN TABLE 5

Batch of lactalbumin hydrolysate	A	B	C	D	E	F	G	H
A	0.00							
B	120.10*	0.64						
C	15.58*	48.70*	0.00					
D	9.09*	62.33*	0.64	0.64				
E	9.09*	62.33*	0.64	0.00	0.64			
F	86.36*	2.59	28.57*	38.96*	38.96*	0.64		
G	22.07*	38.96*	5.84*	2.59	0.00	20.77*	0.00	
H	13.63*	48.70*	0.81	1.94	1.94	23.77*	0.00	0.64

*Each F_{cal} value is based on a comparison of the average value of the duplicate flasks for a batch with the corresponding average for the other batch with which it is being compared. The F_{cal} value resulting from comparing one flask with another in a pair is, of course, an exception to this statement. Any value for F_{cal} greater than 3.95 means that the difference between the two samples is significant at the 95% confidence level.

each media. The results (Fig. 3, experiment 1), indicated that 5 and 7.5% FBS were insufficient for the best growth. Consequently, 10% FBS was used in the formulation of medium C.

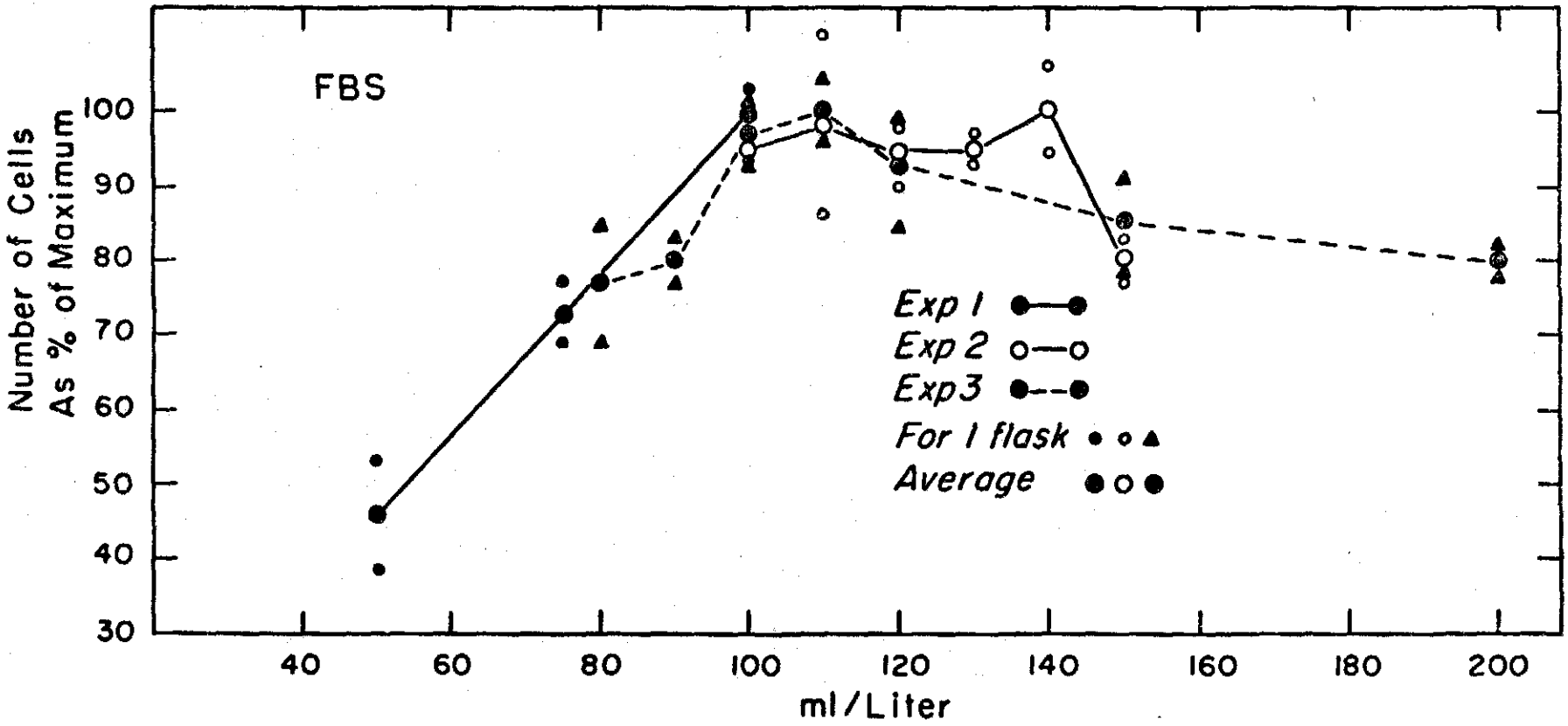
Once medium C was prepared, concentrations of FBS higher than 10% were studied. The effect of 10, 11, 12, 13, 14 and 15% FBS on the growth of AC20 cells was also measured by cell counts in the phase contrast microscope. The results (Fig. 3, experiment 2), indicated that there was no difference in the growth of AC20 cells when the FBS concentration was increased from 10% to 14%. There was a slight decrease in the growth with 15% FBS. A third experiment tested concentrations of 8, 9, 10, 11, 12, 15 and 20% FBS. Here again concentrations below 10% indicated that they were not sufficient for the best growth of AC20 cells. There was no difference between 10, 11 and 12% FBS, but at concentrations of 15% or 20% there was a reduction in the growth of the cells (Fig. 3, experiment 3).

These results and earlier studies with FBS indicated that a concentration of 10% FBS is required for the best growth of these cells. There seems to be an optimal plateau region between 10 and 14% FBS. Higher concentrations made the medium more expensive but did not provide better growth.

C. Suitability of Different Batches of Fetal Bovine Serum

Since the first work in this laboratory on tissue culture of agallian cells, it has been observed that different batches of FBS differ in their capacity to support growth in the media used. At first the selection of a good batch of FBS was made by observing under the phase contrast microscope, cells growing in media prepared with different samples of FBS. The batch of FBS that appeared to give cells in the best condition was selected. Later the Coulter Counter permitted quantification of some of the differences

Fig. 3. The effect of various concentrations of fetal bovine serum (FBS) in ml/liter of medium on the growth of AC20 cells. Each small point represents the count for one flask of the pair, and each large point the average of the pair, representing one treatment in an experiment. All averages were plotted as a percentage of the maximum average in an experiment. The curve shows that there is an optimal concentration of FBS of about 11%.


FBS
Number of Cells
As % of Maximum
100
90
80
70
60
50
40
30
40
60
80
100
120
140
160
180
200
ml/Liter
Exp 1
Exp 2
Exp3
For 1 flask
Average

observed under the microscope, and for a time this technique was used in the selection of suitable batches of FBS. However, the differences observed in the numbers of cells were very small and it was necessary to use at least four replicate flasks for each one of the samples tested, and to repeat the test several times, before a batch that had been consistently good could be selected.

When the counting technique with the phase contrast microscope was developed, a larger number of batches could be tested readily and the possibility of selecting a good batch of FBS was increased. This technique was used to test 12 batches of FBS from different commercial suppliers. Some samples were toxic to the cells as early as 24 hours after subculture. Most of the batches from some companies were not toxic, but they varied in their suitability for supporting growth of the AC20 cells. In all cases but one, the growth ranged from 50 to 79% of the control used, a batch of FBS selected one year earlier. One sample tested was at least 20% better than the control and this batch was used as a source of FBS in the formulation of media C and D (Table 1).

Nine samples of FBS produced under the supervision of a colleague and supplied by him as a courtesy were compared with the best commercial sample selected in these earlier tests. None of these samples was as good as this control batch of commercial FBS but most of them were better than any of the other eleven commercial batches. In these tests it was again observed that there were differences in the suitability of different samples of FBS for the culture medium for agallian cells. The growth of AC20 cells in the media prepared with these nine samples ranged from 55 to 86% of the control.

D. Heat Treatment of Fetal Bovine Serum

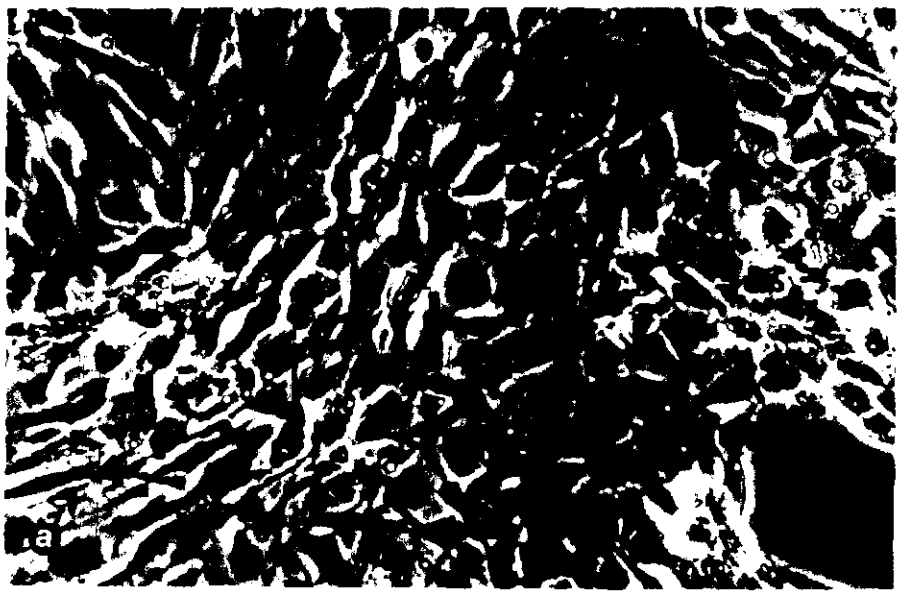
As a regular procedure described in "Materials and Methods," FBS was heat-treated by bringing it quickly to 56°C immediately after it was thawed, holding it at that temperature for 30 minutes and then cooling it to 0°C. The need for heat treatment was tested by comparing heat-treated and non-heat treated aliquots of 9 different samples of FBS and the FBS control. It was observed that 24 hours after subculture, insect cells in 7 of 9 samples that were not heat treated were in very poor condition with much cell debris and many detached cells. Cells growing in media prepared with the other two samples were not in quite such poor condition. Only the unheated control FBS (Fig. 4) supported growth sufficient to justify cell counts and these showed that it yielded only 50% of the growth in the medium prepared with the heated control FBS.

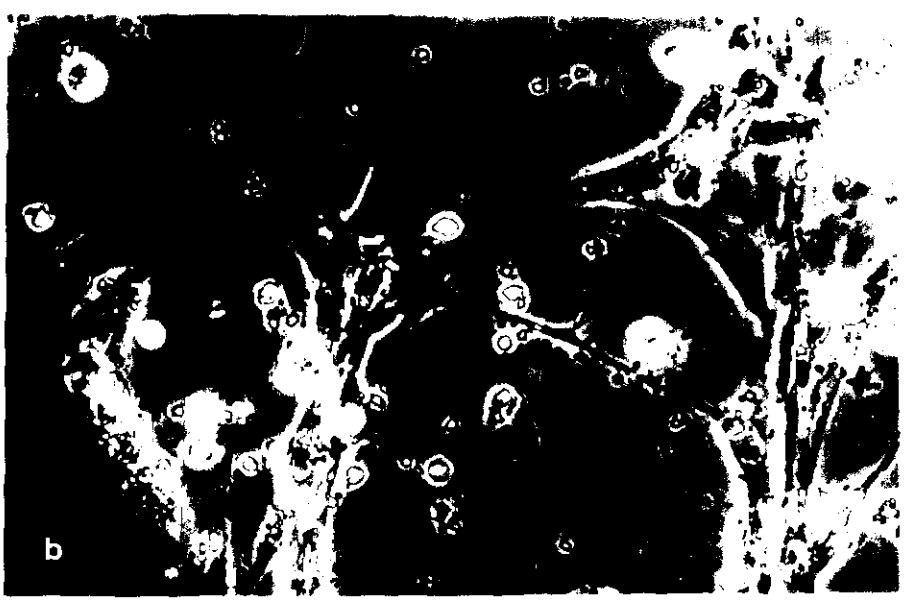
E. Optimal pH

Even though accurate buffering of media used to grow insect cells *in vitro* is desirable (Vago, 1967), very few studies have determined optimal pH. In general the pH of the media for insect tissue culture has been determined by measurements of the pH of the hemolymph of the species to be grown (Wyatt *et al*, 1956; Vago and Flandre, 1963). A phosphate buffer system with a range from 6.3 to 7.4 has been the most widely used (Wyatt, 1956; Martignoni and Scallion, 1961; Grace, 1962; Hirumi and Maramorosch, 1964; Chiu and Black, 1967; Vago, 1967).

Earlier studies with medium 0 (Chiu and Black, 1967) indicated that the buffering capacity of this medium was poor at pH 7.1 (Schlegel and Black, 1969), the pH considered at that time to be the optimal. However, because it was difficult to maintain the pH of this medium at any pH value between 6.0

Fig. 4. a) AC20 cells in medium B with heat-treated FBS. b) AC20 cells in medium B with FBS that had not been heat-treated. Note the poorer growth, greater number of globules and greater amount of debris in Fig. 4b than in Fig. 4a. (Phase contrast microscopy, x310)


a


b

and 7.0, information about the optimal pH was difficult to obtain. When experiments were made adjusting the pH of the medium to values between 6.0 and 7.0, the pH of the medium changed during Millipore filtration, and increased during storage of the sterile medium at room temperature or in the cold, to reach values as high as pH 7.5.

Because of the success of using a buffer solution containing 0.1 M histidine and 0.01 M $MgCl_2$ for inoculations of AC cells with WTV (Kimura and Black, 1971), histidine was considered as a means of buffering for medium A. L-histidine-HCl (monohydrate)--L-histidine (free base) were mixed in the combinations required to make medium A with a pH of 7.0, the pH then considered close to the optimum, with concentrations of 0.3, 0.15, 0.075, 0.03, 0.01 and 0.005 M histidine. When these media were used to grow the cells, cells growing in the medium with 0.3 M histidine detached in less than 24 hours. Cells in medium with 0.15 M histidine showed poor growth, and this concentration was considered too great for growth of the cells.

At this time measurements of growth were made using the Coulter Cell Counter to count cells in samples from each one of the treatments. Because no differences were observed in growth at any concentrations of histidine between 0.075 and 0.005 M, 0.075 M histidine was selected to prepare media with a range of pHs between 5.8 and 7.4. This concentration of histidine had a strong buffering capacity and there were no changes in the pH of the respective media after several months of storage at about $4^{\circ}C$. Apparently the strong buffering capacity of 0.075 M histidine revealed effects of small differences in pH that were not observed when using a medium with a low buffering capacity, e.g. medium A.

Several experiments were performed to determine the optimum pH for the growth of the leafhopper cells (Fig. 5). In all of these experiments the optimum pH for the growth of AC20 cells was close to 6.43. In these experiments, in which growth was measured by means of the Coulter Counter, special care had to be taken to insure good cell dissociation by the trypsin treatment. Cells were more difficult to dissociate in medium with histidine and in several of the experiments the results of the counts with the Coulter Counter were widely at variance with the observations of the cultures with the phase contrast microscope. When the size distribution of the cells was considered, it was observed that a higher percentage of large cells were counted when the cells had been grown in medium with histidine. It was shown that this was due to the presence of a higher proportion of two and three cell aggregates in the samples of cells grown in the medium with histidine. Because longer treatments with trypsin did not separate the cells better, another method for the measurement of the growth of the cells was sought. This led to the development of the counting technique with the phase contrast microscope described in "Materials and Methods."

The optimal pH of the medium was restudied after the most favorable concentration of histidine and the optimal proportion of each one of the components of the medium had been determined. The optimal pH of medium D for the growth of AC cells remained close to 6.43 (Fig. 6).

Preliminary studies were made in the determination of the optimal pH of the medium for the growth of AS and AN cells. These studies indicated that the optimal pH for the growth of AS cells was close to 6.3, whereas the optimal pH for the growth of AN cells was close to 6.4.

Fig. 5. Optimal pH for growth of AC20 cells when medium A was buffered with 0.075 M histidine. In experiments 1 and 2, each solid circle represents the count for one flask of the pair, and each open circle the average of the pair, representing one treatment in an experiment. All averages were plotted as a percentage of the highest single count in an experiment. In experiment 3 and 4 there were 4 and 3 flasks respectively in each treatment. Where the average coincided with one of the single counts both are represented by one solid circle. The optimal pH was close to pH 6.43.

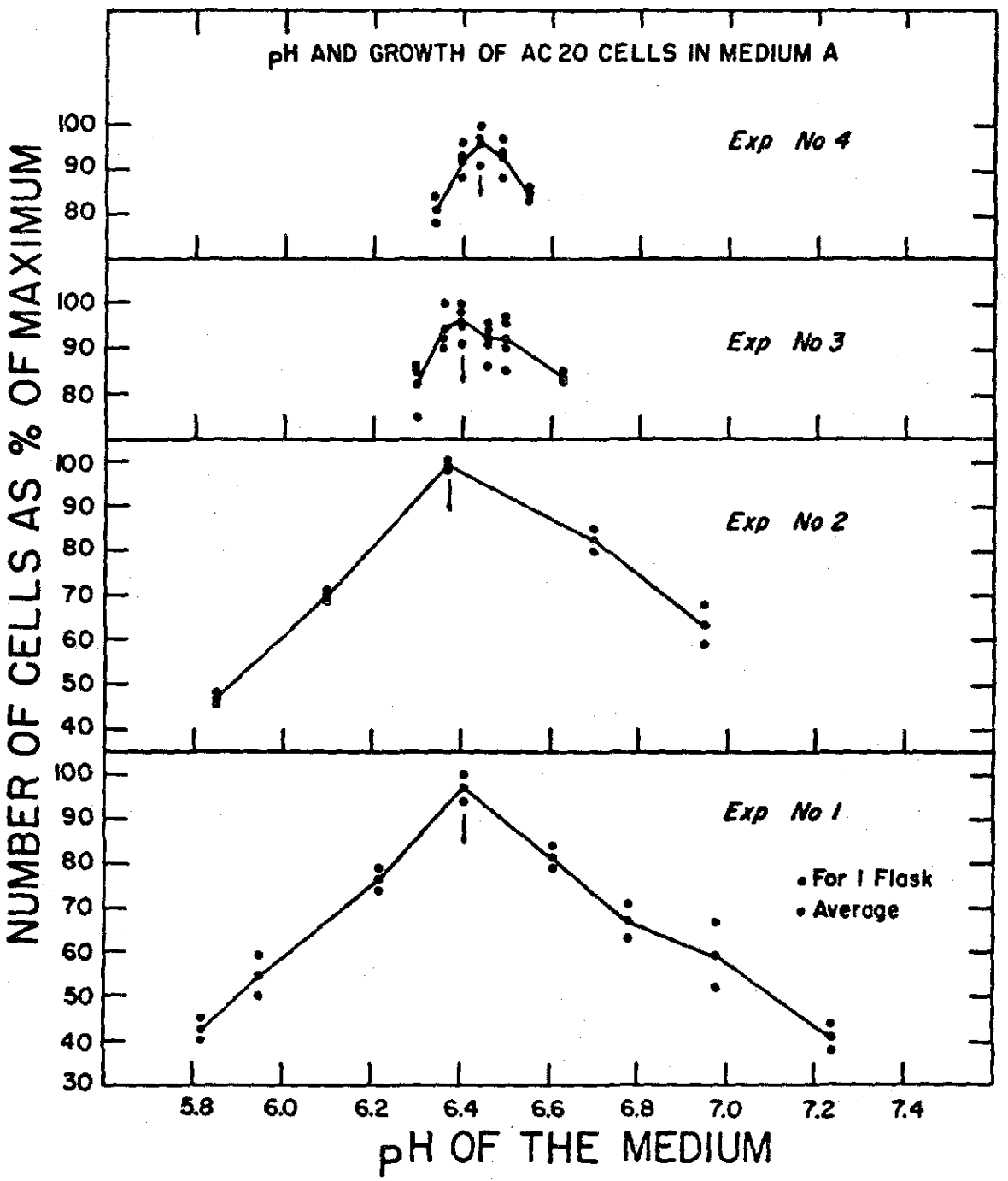
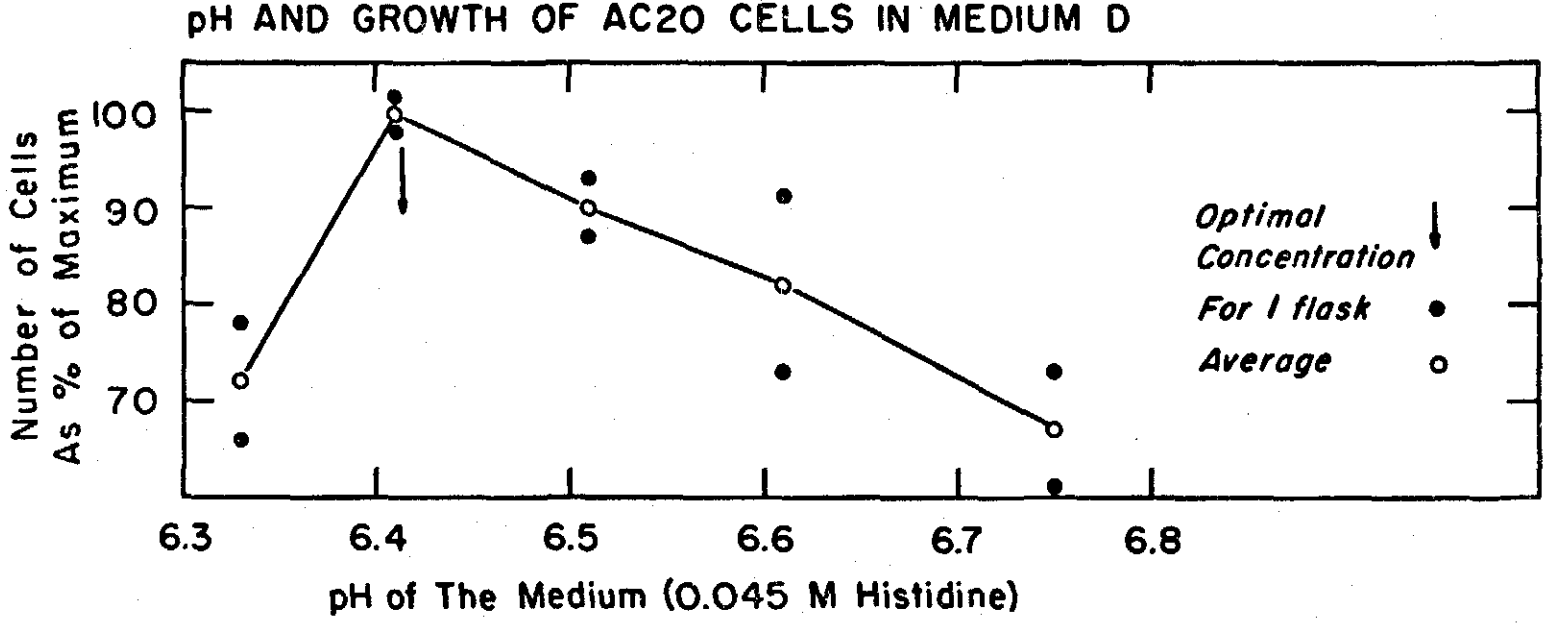

pH AND GROWTH OF AC 20 CELLS IN MEDIUM A
Exp No 4
Exp No 3
Exp No 2
Exp No 1
• For 1 Flask
• Average
NUMBER OF CELLS AS % OF MAXIMUM
pH OF THE MEDIUM
100
90
80
70
60
50
40
30
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4

Fig. 6. Optimal pH for growth of AC20 cells in medium D buffered with 0.045 M histidine.


pH AND GROWTH OF AC20 CELLS IN MEDIUM D
Number of Cells
As % of Maximum
100
90
80
70
6.3
6.4
6.5
6.6
6.7
6.8
pH of The Medium (0.045 M Histidine)
Optimal Concentration
For I flask
Average

F. Optimal Concentration of Histidine

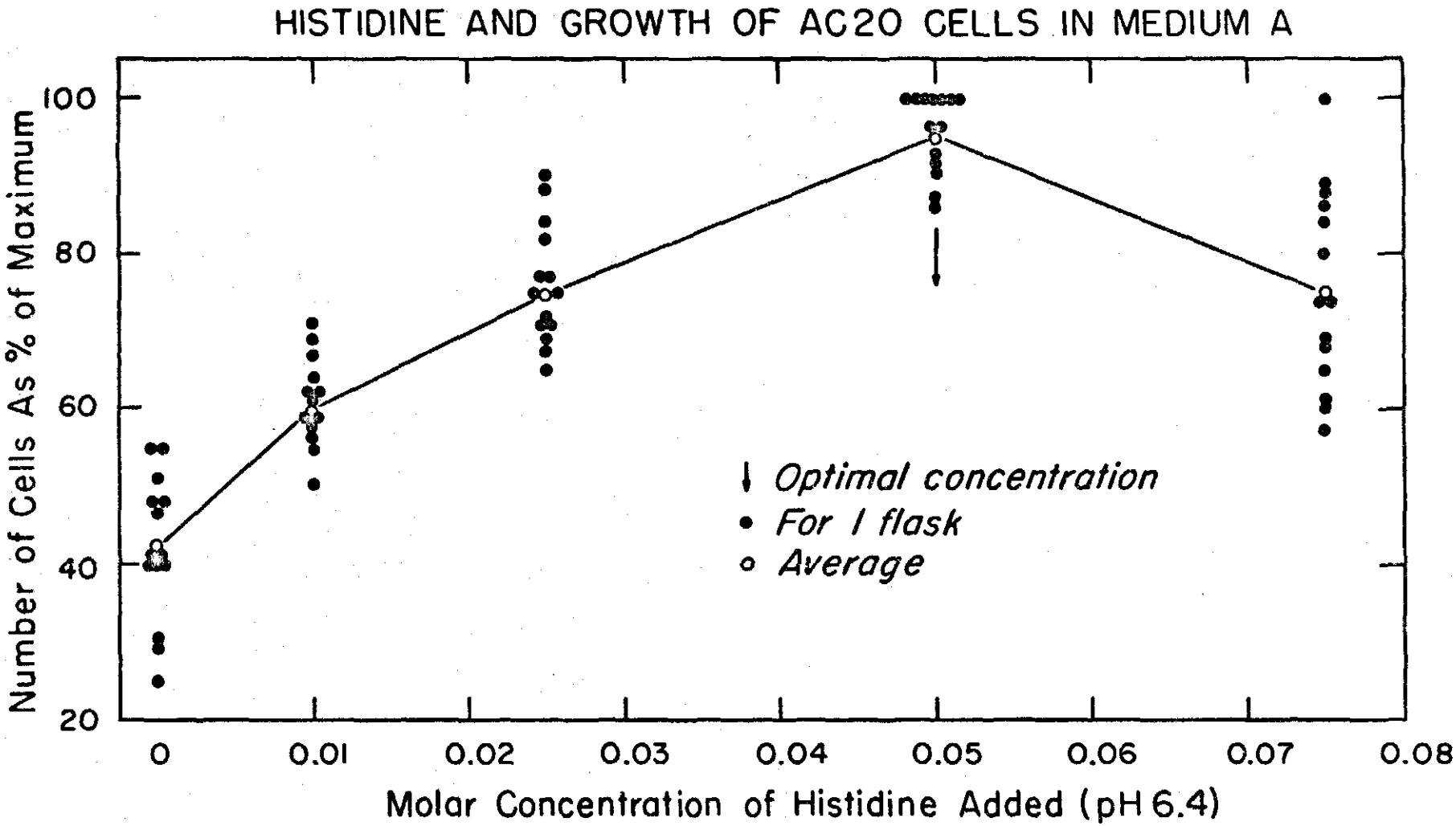
Once the optimal pH for the growth of AC cells was determined by using media with a concentration of 0.075 M histidine, the optimal concentration of histidine was determined. Medium A was prepared, and histidine was added to give concentrations of 0.01, 0.025, 0.05 and 0.075 M histidine at pH 6.43. Increasing concentrations of histidine up to 0.05 M gave faster growth of AC20 cells, but concentrations higher than 0.05 M, e.g. 0.075 M, caused a reduction in growth (Fig. 7). The optimal concentration of 0.05 M histidine at pH 6.43 was obtained by adding 4720 mg L-histidine-HCl and 4270 mg histidine (free base) to every liter of medium. Medium buffered at pH 6.43 with 0.05 M histidine was designated medium B (Table 1).

Medium B was superior to medium O and to medium A; thus, it was used for subculturing all leafhopper cell lines available in the laboratory. AC20 cells were subcultured in this medium through at least 100 passages in series; AC16-2 cells were subcultured through at least 19 passages, and AC18 cells through more than 40 passages. Because the optimal pH for growth of AS cells appeared closer to 6.3 than to 6.43, medium B was prepared at pH 6.3, and AS cells were subcultured in series in medium B (pH 6.3) at least 52 times.

Windsor (1972) found advantages in the use of medium B for the growth of a cell line of AN, a cell line that has been subcultured in series in this medium at least 25 times. Medium B was also used by her in the establishment of primary cultures from a colony of A. novella in which more than 99% of the individuals were carrying the pathogen of Clover Club Leaf Disease.

After the best concentration of each one of the medium components had been determined, batches of medium D at pH 6.43 were prepared with different

Fig. 7. Optimal concentration of histidine for growth of AC20 cells in medium A.


HISTIDINE AND GROWTH OF AC20 CELLS IN MEDIUM A
Number of Cells As % of Maximum
100
80
60
40
20
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
Molar Concentration of Histidine Added (pH 6.4)
Optimal concentration
For 1 flask
Average

concentrations of histidine and these were tested in two experiments. A concentration of 0.045 M histidine was estimated to be optimal (Fig. 8). Table 8 shows the amounts of histidine required for a given pH value between 5.84 and 7.29 when tests were made on medium D with the selected batches of the different components now being used in the laboratory. One may readily plot the values in the table graphically and determine intermediate values from a smooth line giving a good fit with the points. For most of its length such a line is linear. The line changed position somewhat with different quantities and batches of components and should be reconstructed whenever these are changed.

G. Suitability of Different Batches of Histidine

The suitability of batches of L-histidine HCl (monohydrate) and L-histidine (free base) from several commercial suppliers was tested. Medium B was prepared with at least one batch from each one of the suppliers and the media obtained used to grow AC20 cells. Measurement of cell growth was made by counts under the phase contrast microscope. In general there were no big differences in the rate of growth observed with each one of the samples tested, but the sample from one of the suppliers gave poor growth and was considered not to be suitable for a culture medium for agallian leafhopper cells. The growth with this sample was only 74% of the growth obtained with the best batch. The best batch was selected for the preparation of all media tested in further experiments. The other samples were within a range of 84 to 98% of the best, differences that did not appear to be significant.

Fig. 8. Optimal concentration of histidine for growth of AC20 cells in medium D.

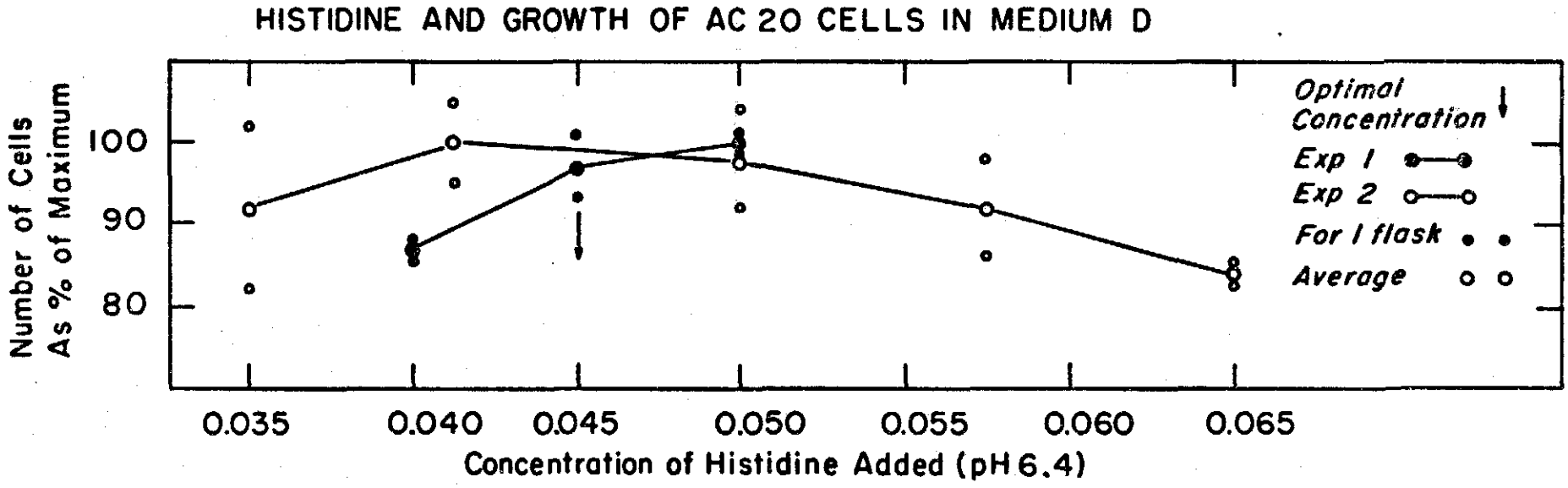

HISTIDINE AND GROWTH OF AC 20 CELLS IN MEDIUM D
Number of Cells As % of Maximum
100
90
80
0.035
0.040
0.045
0.050
0.055
0.060
0.065
Concentration of Histidine Added (pH 6.4)
Optimal Concentration
Exp 1
Exp 2
For 1 flask
Average

Table 8

DIFFERENT pH VALUES IN MEDIUM D WITH COMBINATIONS OF HISTIDINES FOR A FINAL MOLAR CONCENTRATION OF 0.045

Histidine HCl (monohydrate)		Histidine (free base)		pH*
Molar concentration	mg/liter	Molar concentration	mg/liter	
0.0450	9,432	0.0000	0	5.84
0.0405	8,488	0.0045	698	6.00
0.0360	7,545	0.0090	1,396	6.12
0.0315	6,602	0.0135	2,095	6.20
0.0270	5,659	0.0180	2,793	6.34
0.0225	4,716	0.0225	3,492	6.45
0.0180	3,772	0.0270	4,190	6.58
0.0135	2,829	0.0315	4,888	6.74
0.0090	1,886	0.0360	5,587	6.91
0.0045	943	0.0405	6,285	7.06
0.0000	0	0.0450	6,984	7.29

*pH values may change somewhat with different batches of components.

H. Changes in the pH of the Medium

During the experiments to determine optimal pH for growth of AC cells, an increase in the pH of both media O and A during storage was observed at both room temperature and at 4°C. The pH changes were not uniform in different containers of a single batch of medium. When the medium was buffered with histidine (medium B, B_1, C or D of Table 1) all such changes in pH were eliminated.

When changes in pH of the medium occurred in flasks with AC20 cells, it was observed that the change during the first 4 or 5 days after subculture always occurred in the direction which brought the medium closer to the optimal pH for cell growth no matter on which side of the optimum the initial pH lay. After this initial change it was observed that the pH of the medium increased with time at a faster rate in unbuffered than in histidine buffered medium. The changes in pH on long term observations were made with cells stored at 13°C. Tables 9 and 10 present the changes in pH observed either at 28-29°C or during storage at 13°C. Changes during cell growth were more conspicuous when the initial pH of the medium was higher than optimal. On the other hand, when the pH of the medium was lower than the optimal, the changes of pH were smaller and adverse effects on the growth of the cells was more severe. The tendency of pH of the medium to change toward the optimum was also observed during the growth of AS and AN cells (Table 9 and 10).

After the formulation of medium D, pH measurements on medium that had been supporting growth of AC20 cells indicated that during a 4 day period there were no major changes in pH of the medium when the original pH was 6.4.

CHANGES IN THE pH OF THE MEDIUM SUPPORTING GROWTH OF AGALLIAN LEAFHOPPERS AT 28-29°C

Number of samples	Initial pH of the medium	Molar concentration of histidine	Final pH Average	Final pH Range	Days after subculture
A. constricta					
3	7.23	0.000	7.06	7.05-7.07	1
3	7.23	0.000	6.79	6.78-6.81	2
6	7.11	0.000	7.00	6.94-7.13	1
9	7.11	0.000	6.88	6.64-6.99	2
5	7.11	0.000	6.77	6.68-6.83	3
12	7.11	0.000	6.73	6.41-6.70	4
11	6.71	0.000	6.49	6.37-6.61	4
13	6.54	0.000	6.49	6.32-6.61	4
3	6.13	0.000	6.44	6.44	4
3	6.96	0.030	6.92	6.92-6.93	1
3	6.96	0.030	6.80	6.73-6.84	2
2	6.96	0.030	6.76	6.75-6.76	3
7	6.40	0.050	6.44	6.40-6.48	4
3	7.31	0.075	7.08	7.07-7.09	2
3	7.31	0.075	6.89	6.85-6.92	4
3	7.22	0.075	7.05	7.01-7.07	2
5	7.22	0.075	6.88	6.83-6.93	4
3	7.11	0.075	6.99	6.97-7.01	2
3	7.11	0.075	6.84	6.80-6.87	4
3	6.97	0.075	6.92	6.87-6.96	2
5	6.97	0.075	6.78	6.75-6.80	4
3	6.80	0.075	6.78	6.76-6.80	2
5	6.80	0.075	6.68	6.66-6.69	4
3	6.62	0.075	6.63	6.62-6.65	2
5	6.62	0.075	6.59	6.57-6.64	4
3	6.40	0.075	6.43	6.42-6.43	2
6	6.40	0.075	6.41	6.38-6.45	4
3	6.22	0.075	6.25	6.25	2
5	6.22	0.075	6.24	6.20-6.27	4
2	5.95	0.075	6.00	5.98-6.01	2
2	5.82	0.075	5.84	5.83-5.84	4
1	6.95	0.150	6.95	6.95	1
1	6.95	0.150	6.91	6.91	2
A. sanguinolenta					
6	6.73	0.000	6.32	6.22-6.41	4
3	6.39	0.000	6.36	6.35-6.37	4
2	6.23	0.000	6.26	6.25-6.27	4
1	6.05	0.000	6.10	6.10	4
3	6.43	0.005	6.31	6.29-6.33	4
3	6.40	0.010	6.28	6.28	4
16	6.38	0.050	6.35	6.29-6.41	4
3	6.52	0.100	6.47	6.46-6.48	4
3	6.36	0.100	6.34	6.33-6.34	4
3	6.13	0.100	6.13	6.13	4
A. novella					
7	6.81	0.000	6.52	6.38-6.67	4
11	6.41	0.050	6.41	6.39-6.44	4

Table 10

CHANGES IN THE pH OF THE MEDIUM SUPPORTING GROWTH OF AGALLIAN LEAFHOPPERS DURING STORAGE OF THE CELLS AT 13°C

Number of samples	Initial pH of the medium	Molar concentration of histidine	Final pH Average	Range	Days of storage
A. constricta					
5	6.40	0.000	6.77	6.67-7.03	30
5	6.40	0.000	6.84	6.79-6.96	50
7	6.40	0.000	7.13	6.86-7.38	120
15	6.40	0.000	7.62	7.51-7.72	150
4	6.40	0.050	6.42	6.40-6.46	30
4	6.40	0.050	6.45	6.41-6.50	50
8	6.40	0.050	6.50	6.42-6.75	60
7	6.40	0.050	6.60	6.51-6.82	75
17	6.40	0.050	6.52	6.31-6.62	120
4	6.40	0.050	6.48	6.47-6.48	135
30	6.40	0.050	6.75	6.53-7.07	150
A. sanguinolenta					
4	6.30	0.000	6.76	6.62-6.83	80
6	6.30	0.050	6.29	6.27-6.34	80
A. novella					
3	6.40	0.000	7.32	7.15-7.38	140
7	6.40	0.050	6.87	6.73-6.90	140

I. Comparisons of Medium B with Other Tissue Culture Media

1. Grace's insect tissue culture medium

The rate of growth of AC20 cells in medium B was compared with the growth of the cells in Grace's insect tissue culture medium (1962) (Grand Island Biological Co., Grand Island, New York). At the same time concentrations of 10 and 20% Grace's medium were incorporated into medium B and the effect on the growth of the cells measured with the phase contrast microscope. Cells growing in Grace's medium yielded only 51% of the growth in medium B. When 10% Grace's medium was used there was a reduction in the rate of growth to 85% of the growth in medium B. This reduction was greater, only 75% of the growth in medium B, when 20% Grace's medium was used.

2. Schneider's Drosophila tissue culture medium

One experiment was done in which the growth of AC20 cells in medium B was compared with the growth in Schneider's revised insect tissue culture (1964) (Grand Island Biological Co.) and in medium B in which 10 or 20% Schneider's medium was incorporated. Results indicated that AC20 cells were not able to grow in Schneider's medium. Twenty-four hours after subculture most of the cells in Schneider's medium were detached and there was a large amount of debris from ruptured cells. When 10 and 20% Schneider's medium were incorporated in medium B the growth was 73% and 52%, respectively, that of the growth in medium B.

3. Mitsuhashi and Maramorosch's leafhopper tissue culture medium

The growth supporting capacity of media B and C for AC20 cells was compared with the growth in Mitsuhashi and Maramorosch's leafhopper tissue culture medium (1964). This medium was prepared with the same batches of components used in the preparation of media B, C and D (Table 1). The

results indicated that AC20 cells were unable to grow satisfactorily in Mitsuhashi and Maramorosch's medium. When 10 and 20% of this medium were incorporated into medium B the growth was respectively, 90% and 72% of the growth in medium B alone. In this test the growth of AC20 cells in medium B was 67% of the growth in medium C.

4. Other tissue culture media

One experiment was performed in which the growth supporting capacity of medium B was compared with the growth of AC20 cells in NCTC-135 medium (Evans et al, 1956) (Grand Island Biological Co.). It was observed that after 24 hours of subculture in NCTC-135 medium all AC20 cells appeared to be dead. When 10 and 20% of NCTC-135 medium were added to medium B there were reductions of 20 and 33%, respectively, of the growth in medium B.

Similar results to those obtained with NCTC-135 medium were observed when AC20 cells were grown in BHK21 medium (MacPherson and Stoker, 1962) (Grand Island Biological Co.) supplemented with 10% calf serum (not heat-treated), 10% tryptose phosphate and 1% antibiotic antimycotic solution (100x) (Grand Island Biological Co.) or in MEM medium with Earle's salts (Eagle, 1959) (Grand Island Biological Co.) supplemented with 10% calf serum (not heat-treated), 10% tryptose phosphate and 1% antibiotic-antimycotic (100x) (Grand Island Biological Co.). Cells growing in any of these two media appeared to be dead in less than 24 hours after subculture.

J. Optimal Concentration of Each One of the Medium Components

Each one of the components used in the preparation of medium B, except FBS and histidine, was incorporated at different concentrations in different batches of medium B (pH 6.43) in which all other components were kept at the concentration given in Table 1. FBS, histidine and pH were each maintained

at the same concentration throughout, a concentration that seemed to be a stable optimum despite the other changes. Batches varying in the one component were tested together for their relative ability to support the growth of AC20 cells. Measures of growth were plotted for each flask and an estimate of the optimal concentration of the component was made on the basis of the shape of the curve obtained.

Based on the results of the first set of tests on each component, medium C was prepared with suitable newly selected concentrations of each component and in the light of information on favorable osmotic pressures for the medium. Each component was then tested again in medium C by the same procedure as before. A few additional tests had to be made with a few of the components before making what was hoped would be a final formulation, namely, that of medium D.

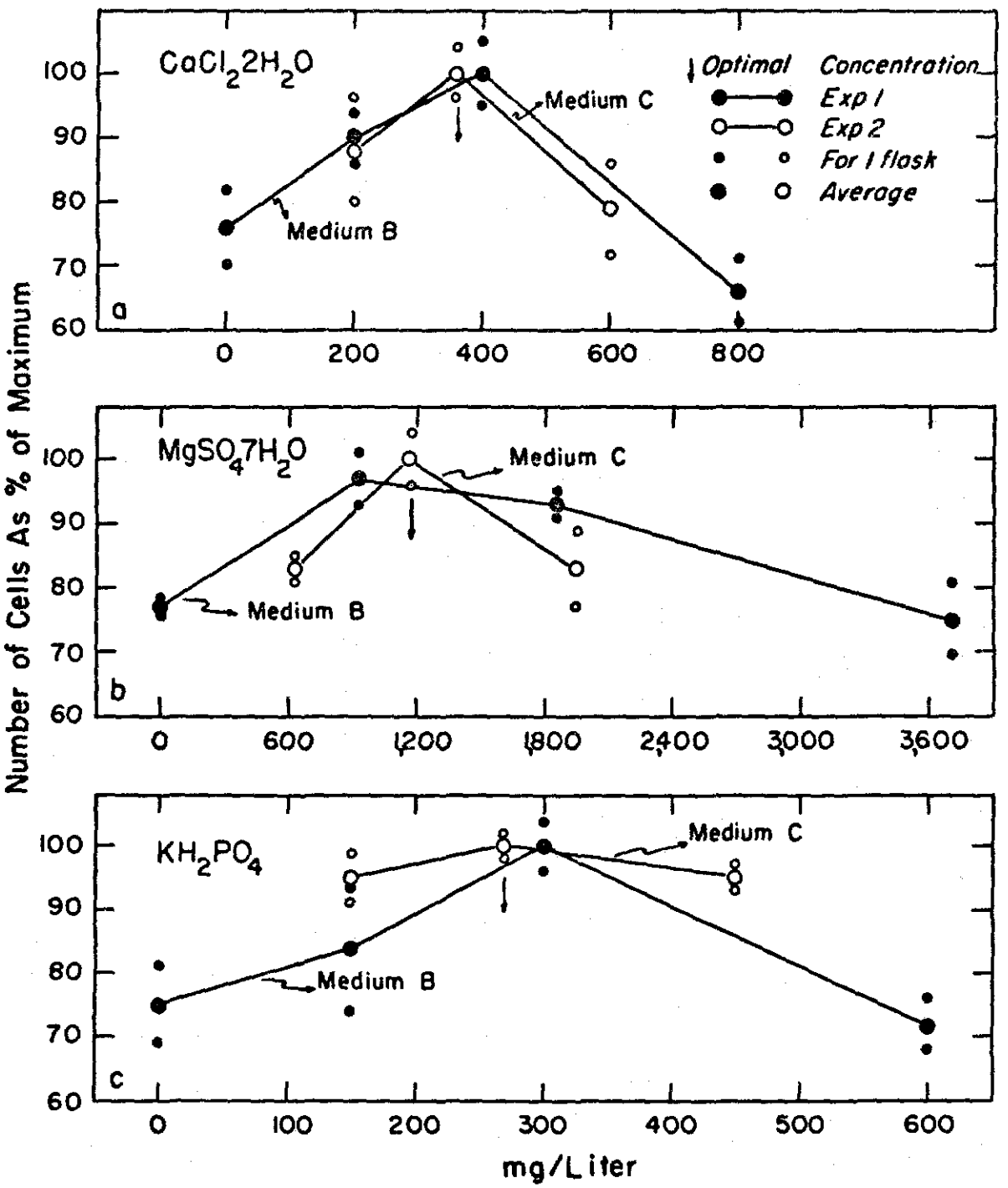
1. Optimal concentration of calcium chloride

Modified batches of medium B (Table 1) were prepared so that the concentration of $CaCl_2.2H_2O$ was either 0, 200, 400 or 800 mg per liter. The growth measured by counts of nuclei showed that the concentration of 400 mg/liter was close to the optimum (Fig. 9a, experiment 1). A second test was made using concentrations of 200, 360 and 600 mg of $CaCl_2.2H_2O$ per liter in batches of medium C (Table 1). The optimal concentration of $CaCl_2.2H_2O$ was close to 360 mg/liter (Fig. 9a, experiment 2).

2. Optimal concentration of magnesium sulfate

AC20 cell growth was tested in medium B with 0, 926, 1,853 and 3,706 mg $MgSO_4.7H_2O$ per liter. The results indicated that the optimal concentration was near 1,300 mg/liter (Fig. 9b, experiment 1). A second test was made using medium C with 650, 1,170 and 1,950 mg $MgSO_4.7H_2O$ per liter

Fig. 9. a) Optimal concentration of $CaCl_2.2H_2O$ in medium B and in medium C. b) Optimal concentration of $MgSO_4.7H_2O$ in medium B and in medium C. c) Optimal concentration of KH_2PO_4 in medium B and in medium C.


CaCl2 2H2O
Medium C
Medium B
Optimal Concentration
Exp 1
Exp 2
For 1 flask
Average
a
MgSO4 7H2O
Medium C
Medium B
b
KH2PO4
Medium C
Medium B
c
Number of Cells As % of Maximum
mg/Liter

and a concentration of 1,170 mg/liter gave the best growth (Fig. 9b, experiment 2).

3. Optimal concentration of potassium phosphate

The optimal concentration of KH_2PO_4 was investigated in medium B with 0, 150, 300 and 600 mg KH_2PO_4 per liter. Counts of AC20 nuclei indicated that 300 mg/liter was close to the optimum (Fig. 9c, experiment 1). After formulation of medium C, concentrations of 150, 270 or 450 mg KH_2PO_4 per liter in modified batches of medium C were tested and a concentration of 270 mg of KH_2PO_4 per liter proved close to the optimum (Fig. 9c, experiment 2).

4. Optimal concentration of potassium chloride

After growing AC20 cells in medium B with 0, 400, 800 and 1,600 mg KCl per liter, nuclear counts showed that the best cell growth was obtained with 1,600 mg KCl per liter (Fig. 10a, experiment 1). Later, batches of medium C were modified to contain 800, 1,600 and 2,400 mg KCl per liter and the counts of AC20 nuclei showed that the optimal concentration of KCl in medium C was close to 1,600 mg/liter (Fig. 10a, experiment 2).

5. Optimal concentration of sodium chloride

Medium B containing 0, 500, 1,053 and 2,000 mg NaCl per liter were tested and the concentration of 1,053 mg/liter was close to the optimum (Fig. 10b, experiment 1). Batches of medium C containing 500, 1,000 and 1,500 mg NaCl per liter were tested and the best concentration of NaCl was 1,000 mg/liter (Fig. 10b, experiment 2).

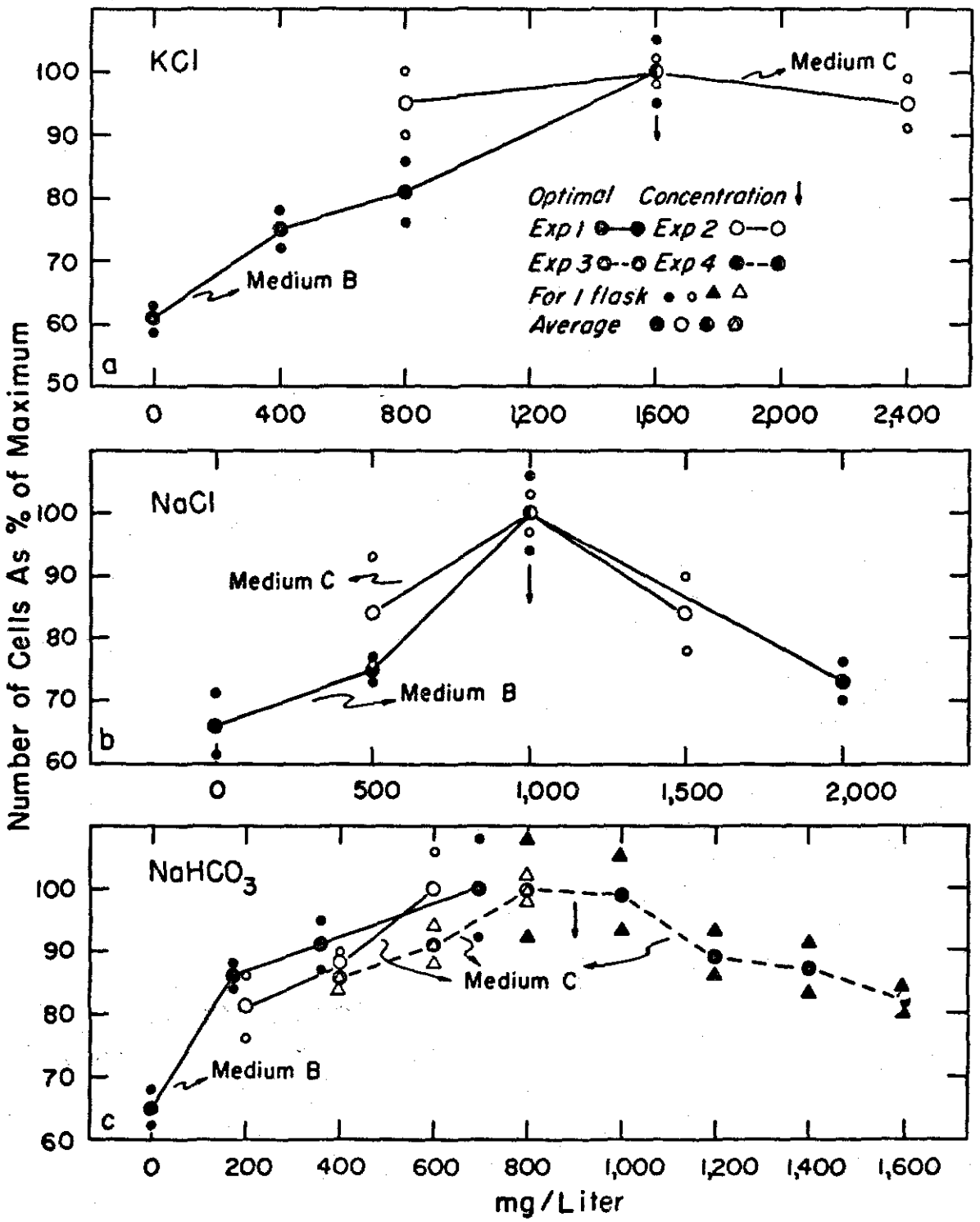
6. Optimal concentration of sodium bicarbonate

Batches of medium B containing 0, 175, 353 and 700 mg $NaHCO_3$ per liter were tested and the optimal concentration of $NaHCO_3$ seemed to be about

Fig. 10. a) Optimal concentration of KCl in medium B and in medium C.

b) Optimal concentration of NaCl in medium B and in medium C.

c) Optimal concentration of $NaHCO_3$ in medium B and in medium C.

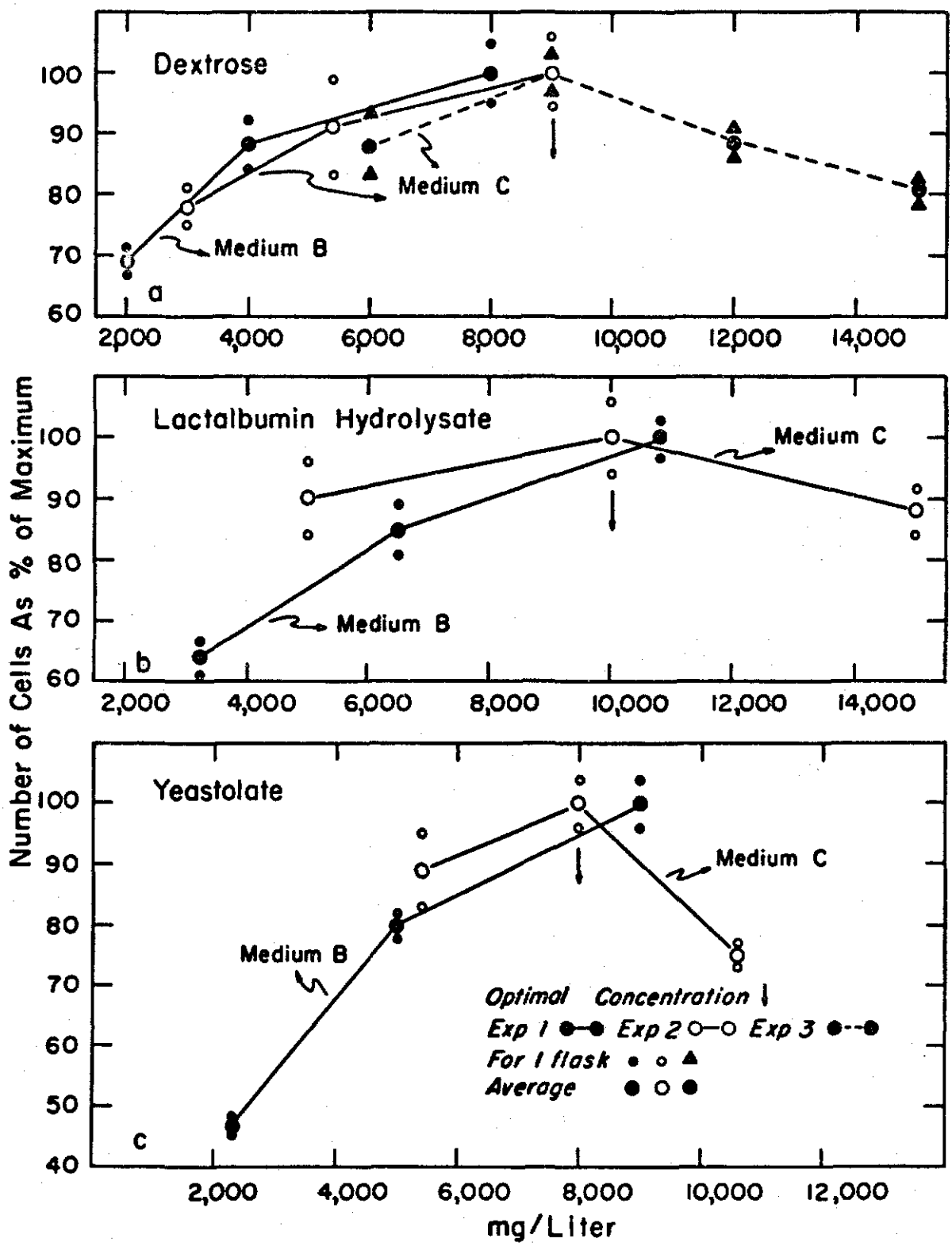

KCl
Medium C
Medium B
Optimal Concentration
Exp 1 Exp 2
Exp 3 Exp 4
For 1 flask
Average
a
NaCl
Medium C
Medium B
b
NaHCO3
Medium C
Medium B
c
Number of Cells As % of Maximum
mg/Liter

700 mg/liter (Fig. 10c, experiment 1). Because of the small differences in growth observed between 353 and 700 mg/liter the concentration of $NaHCO_3$ was only increased to 400 mg/liter when medium C was formulated. When batches of medium C containing 200, 400 and 600 mg $NaHCO_3$ per liter were tested the best growth occurred at the highest concentration (Fig. 10c, experiment 2). In an additional test with a higher range of concentrations of $NaHCO_3$ the highest concentration again gave the best growth (Fig. 10c, experiment 3), an indication that even though the differences in growth were small the optimal concentration was more than twice the concentration in medium B. In a fourth test with $NaHCO_3$, concentrations of 800, 1,000, 1,200, 1,400 and 1,600 mg per liter of medium C indicated that the optimal concentration of $NaHCO_3$ was about 900 mg/liter (Fig. 10c, experiment 4). This concentration was used in the formulation of medium D.

7. Optimal concentration of dextrose

Growth tests were made with batches of medium B containing 2,000, 4,000 and 8,000 mg of dextrose per liter. The highest concentration of dextrose used (8,000 mg/liter) gave the best growth (Fig. 11a, experiment 1). Because of the small differences in growth observed at the two higher dextrose concentrations with medium B and judging by the appearance of the growth curve obtained in this experiment, it was estimated that 6,000 mg/liter would be closer to the optimum than either 4,000 or 8,000 mg/liter. In the formulation of medium C it was decided to reduce the 6,000 mg/liter to 5,400 mg/liter because of the results observed in preliminary experiments on the optimal osmotic pressure of the medium. Growth tests were performed with batches of medium C modified to contain 3,000, 5,400 and 9,000 mg of dextrose per liter. The best concentration for growth was the highest

Fig. 11. a) Optimal concentration of dextrose in medium B and in medium C. b) Optimal concentration of lactalbumin hydrolysate in medium B and in medium C. c) Optimal concentration of yeastolate in medium B and in medium C.


Dextrose
Medium C
Medium B
a
Lactalbumin Hydrolysate
Medium C
Medium B
b
Yeastolate
Medium C
Medium B
Optimal Concentration
Exp 1 Exp 2 Exp 3
For 1 flask
Average
c
Number of Cells As % of Maximum
mg/Liter

concentration used (Fig. 11a, experiment 2). This indicated that even though this amount of dextrose increased the osmotic pressure of the medium it was not certain that 9,000 mg/liter was optimal. In an additional test with concentrations of 6,000, 9,000, 12,000 and 15,000 mg of dextrose per liter the optimal concentration was about 9,000 mg/liter (Fig. 11a, experiment 3).

8. Optimal concentration of lactalbumin hydrolysate

Batches of medium B in which the concentration of lactalbumin hydrolysate was 3,250, 6,500 or 10,800 mg per liter (Fig. 11b, experiment 1) were tested and the best growth was obtained with the highest concentration. As a result, in the formulation of medium C, 10,000 mg of lactalbumin hydrolysate per liter was chosen. Additional tests were made with batches of medium C containing 5,000, 10,000 and 15,000 mg of lactalbumin hydrolysate per liter. The best concentration was 10,000 mg/liter (Fig. 11b, experiment 2).

9. Optimal concentration of yeast hydrolysate

Tests for the optimal concentration of yeast hydrolysate (Yeastolate, DIFCO, Detroit, Michigan) in medium B were made with batches containing 2,225, 5,000 and 9,000 mg of Yeastolate per liter. The highest concentration tested (9,000 mg/liter) gave the best growth (Fig. 11c, experiment 1). It was estimated from these results that a concentration of 8,000 mg/liter might be close to the optimum and this concentration was used in the formulation of medium C. Batches of medium C containing Yeastolate at concentrations of 5,400, 8,000 and 10,600 mg/liter were tested and it was concluded that 8,000 mg/liter was close to the optimum (Fig. 11c, experiment 2).

10. Optimal concentration of antimicrobial agents

Media O, A, B, B_1 and C were prepared with the same concentrations

of antimicrobial agents used by Chiu and Black (1967) in the medium employed to start the cell lines used in these experiments. These components have been added to the medium in two fractions, one of them a Fungizone fraction (Amphotericin B, E. R. Squibb & Sons, Inc., New York, New York) and the other a mixture of Potassium Penicillin G (Eli Lilly and Co., Indianapolis, Indiana), Streptomycin Sulfate (Eli Lilly and Co.), and Neomycin Sulfate, (TRENT Pharmaceuticals, Inc., New York, New York). The concentrations used are given in Table 1.

The effect of these two fractions on the growth of the cells was studied by omitting them from the medium, by using the amounts in media O, A, B, B_1 and C, and by using twice and four times these amounts. No differences in growth rate were detected between any of the concentrations of antibiotics tested. The concentrations used in media O, A, B, B_1 and C (Table 1) were used in the formulation of medium D.

Gentamicin (Schering Corporation, Port Reading, New Jersey), an antibiotic with several advantages over penicillin and streptomycin for use in cell culture (Schafer et al, 1972) was tested in a preliminary experiment. It was incorporated in medium D at concentrations of 0, 50, 100, 250, 500 and 1,000 mg per liter. When growth of AC20 cells was tested in each one of these media, the results indicated that 50 mg/liter gave the best growth and that 1,000, 500, 250, 100 or 0 mg gentamicin per liter gave 38, 79, 86, 98 and 97% of the best growth, respectively. Actually there is no significant difference between the 97, 98 and 100% results. Continuous subculture of AC20 cells in medium D with 50 or 100 mg gentamicin per liter, the effective concentration range, should be conducted to obtain more reliable information about the tolerance of the leafhopper cells to this antibiotic.

Fig. 12. a) AC20 cells growing in medium C with 25 mg of Fungizone per liter. b) AC20 cells growing in medium C with 100 mg of Fungizone per liter. Note the poorer growth and greater number of globules with the greater concentration of Fungizone. (Phase contrast microscopy, x320)

a

b

When different concentrations of Fungizone were tested, medium with 100 mg per liter gave 63% of the growth in the best medium. Besides this reduction in the rate of growth, a large number of the yellowish globules associated with poor growth was observed. When 50 mg per liter was used, growth was 90% of the best growth. This reduction in growth was of uncertain significance, but the cells growing in medium with this concentration of Fungizone still had many of the yellowish globules. No differences in growth were detected between medium without Fungizone and with 25 mg per liter and the latter concentration might be used to advantage in certain circumstances. Ordinarily, however, it seems better to omit Fungizone from the medium. In this connection it may be noted that Stanley and Vaughn (1967) reported that certain insect cells in culture could not be recovered from medium containing 2 mg Fungizone per liter.

K. Supplements to Medium B or C

1. Trehalose

One experiment was made in which d(+) trehalose (Nutritional Biochemicals Corporation, Cleveland, Ohio) was added to medium B at a concentration of 1,000 mg/liter. Cells growing in medium B with trehalose yielded 91% of the growth of cells in medium B, a difference that, though not significant, indicated that the addition of trehalose at this concentration to medium B was not beneficial.

2. Lobster hemolymph

Medium C was supplemented with 1, 2 or 3% lobster hemolymph (Grand Island Biological Co., Grand Island, New York). The lobster hemolymph was heat-treated at 56°C for 30 minutes to render it non-toxic to the cells. During the treatment a precipitate formed which was removed by centrifugation

at 63 g for 5 minutes followed by filtration through a 0.22 μm Millipore filter. The growth of AC20 cells in any of the media prepared with different concentrations of lobster hemolymph was no better than that in medium C.

L. Optimal Osmotic Pressure

There is little information on the osmotic pressure of media for the culture of insect cells. In some media that had been designed from analyses of insect hemolymph, osmotic pressures were usually very close to that of hemolymph (Vaughn, 1971). Wyatt (1956) determined the optimal osmotic pressure for tissue cultures from the silkworm, Bombyx mori L. Even though the method of judging cultures was rather subjective, it was possible to see differences between different dilutions of a medium based on hemolymph analyses. A medium with a freezing point depression of $-0.53^{\circ}C$ (280 mOsm) was considered to be optimal.

Because there are no earlier reports on the determination of optimal osmotic pressure for the growth of agallian leafhopper cells, experiments were designed to ascertain the optimal osmotic pressure of medium for culture of AC20 cells. Medium B was prepared with 2.0x the concentration of each of the components--with the exception of histidine which was kept at a concentration of 0.05 M and was used to maintain the pH of the medium constant at 6.4. Using this hypertonic medium B, dilutions were made with sterile distilled water to give concentrations of 1.66, 1.43, 1.25, 1.11, 1.0, 0.8, 0.6, 0.4 and 0.2x the concentration of components in medium B. These media had osmotic pressures that ranged from 113 to 407 mOsm for the 0.2x to 1.66x concentrations, respectively. Medium with the 2.0x concentration was not used because from earlier experience it was known that AC20 cells were unable to grow at that concentration. When AC20 cells were grown

in each of the media prepared, it was observed that as early as 24 hours after subculture cells in medium with 0.2 or 0.4x concentrations were unable to grow. The growth was also poor at the 0.6x concentration. Four days after subculture, growth at the 0.8x concentration (207 mOsm) was only 60% of the best growth which was obtained at the 1.25x concentration (344 mOsm). Medium B (1.0x) with an osmotic pressure of 273 mOsm gave 88% of the best growth. At osmotic pressures higher than optimal there was of course less than optimal growth, but it was not reduced as much above the optimal concentration as it was below. At 1.66x concentration the growth was 67% of the best growth.

Some modifications were made in medium B in accordance with the results of the experiments for the determination of the optimal concentration of each component (Figs. 3, 9, 10, 11). With this medium (B_1, Table 1) a second test of the osmotic value was designed. To provide a range of osmotic values, media were prepared with concentrations of 1.16, 1.10, 1.02, 1.00, 0.95, 0.90, 0.85 and 0.80x the concentration of medium B_1. Osmotic values from 401 mOsm to 278 mOsm were obtained. The osmotic pressure of the 1.0x medium was 344 mOsm. When AC20 cells were grown in each one of these media the best growth was obtained with the medium with the 0.90x concentration. This medium had an osmotic pressure of 310 mOsm, a value somewhat lower than the optimum observed in the previous test.

Medium C (Table 1) was then prepared. Because of the above result the concentration of dextrose, one of the components with an important role in the osmotic pressure of the medium, was reduced from 6,000 mg/liter in medium B_1 to 5,400 mg/liter in medium C. The following reductions in concentrations of components were also made in preparing medium C: $CaCl_2.2H_2O$

from 400 mg/liter to 360 mg/liter; $MgSO_4.7H_2O$ from 1,300 mg/liter to 1,170 mg/liter; and KH_2PO_4 from 300 mg/liter to 270 mg/liter. All the other components were left at the optimal concentrations. The optimal concentration of each one of the components was tested in medium C (Figs. 3, 9, 10, 11), and some new changes were introduced before medium D (Table 1) was formulated. The optimal osmotic pressure was studied again but with medium D. This medium was prepared with concentrations of 1.12, 1.05, 1.00, 0.96, 0.91 and 0.86x the concentration of components in medium D. Osmotic pressures of 403, 379, 360, 345, 326 and 309 mOsm were obtained. In the preparation of these media, enough FBS was added to give 12% in the medium with the 1.00x concentration of components. This provided concentrations above 10% FBS in the media of less than the 1.0x concentration and avoided the adverse effects on cell growth that have been observed with FBS concentrations below 10%.

When AC20 cells were grown in each one of these media and the effect on the growth measured, the best growth in each of two experiments was obtained with the 1.0x concentration, i.e. medium D, which had an osmotic pressure of 360 mOsm (Fig. 13). When corrections for the differences in the size of the nuclei in the different media were made it was observed that the measured differences between the optimal medium and media with lower osmotic pressures increased, whereas the differences with media with higher osmotic pressures decreased. This optimal osmotic pressure of 360 mOsm corresponded to a freezing point of $-0.67^{\circ}C$ or 11.4 g of NaCl per kilogram of water. This osmotic pressure is a little below the middle of the range of freezing points reported for insect hemolymph, -0.5 to $-0.9^{\circ}C$ (Florkin and Jeaunoux, 1964; Vago, 1967; Vaughn, 1971). The osmotic pressure of 360 mOsm is much higher than the osmotic pressures of any of the media previously used in this

laboratory: 250 mOsm for medium O, 205 mOsm for medium A, 275 mOsm for medium B, 344 mOsm for medium B_1, and 310 mOsm for medium C.

It was observed that cells growing in medium at the optimal osmotic pressure had the least amount of cell debris after subculture.

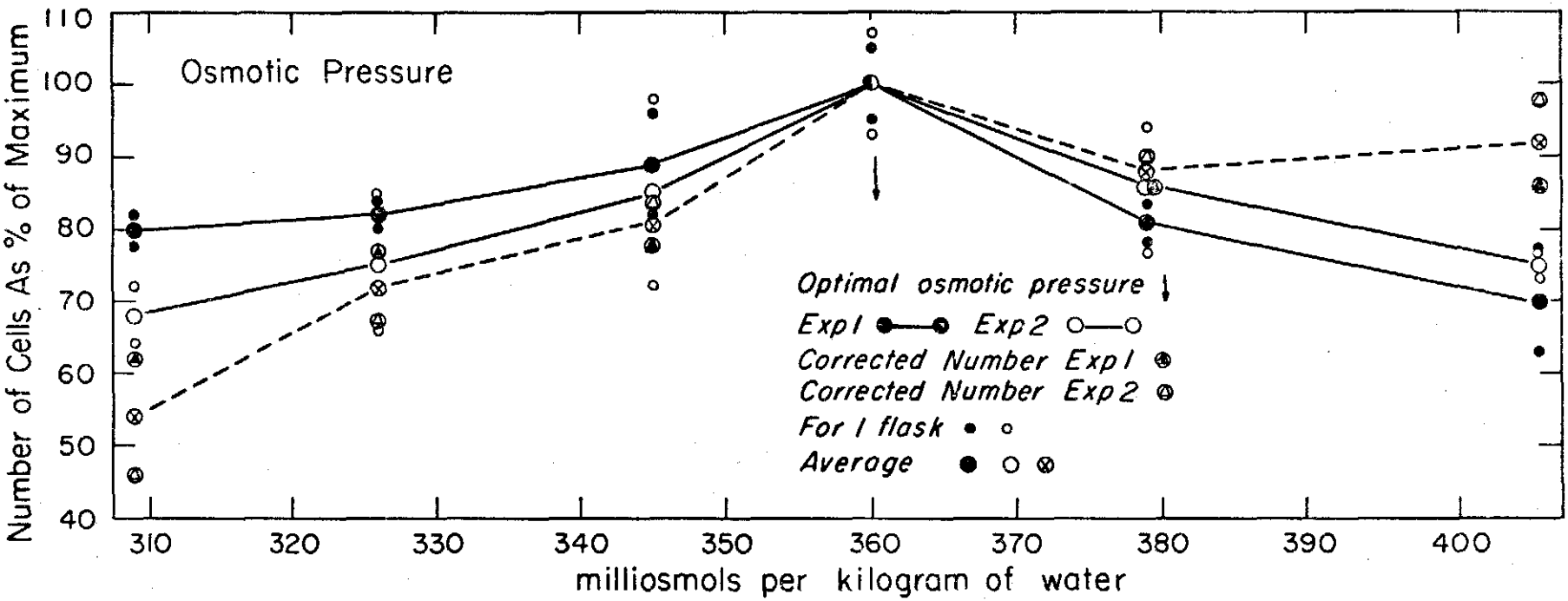
M. Optimal Temperature

1. Optimal temperature for cell growth

The optimal temperature for growth of AC2 in medium O was estimated to be about 27°C by Schlegel and Black (1969). Martinez-Lopez and Black (1971) estimated the optimal temperature for growth of AC20 in medium A to be between 28 and 29°C. The optimal temperature was re-investigated using medium D and the counting technique under the phase contrast microscope.

AC20 cells were subcultured and incubated at about 28°C for 24 hours, then counts were made on each of two flasks to be incubated at 24, 28, 32 or 36°C. After 24 hours of incubation at each temperature a large number of the cells in the flasks at 36°C appeared to be dead and counts of remaining cells showed only 25% as many cells as in the flasks incubated at 28°C, where the best growth occurred. The number of cells at 32°C and 24°C was 78 and 88%, respectively, of the number at 28°C. After 72 hours all the cells in the two flasks at 36°C had detached from the flask. The growth of the cells at 32°C was only 64% of the growth at 28°C, which was still the temperature at which the largest number of cells was counted. At 24°C the growth was 30% of that at 28°C, and here it was possible to see that there was no damage to the cells. When counts were made after 120 hours at the different temperatures, deterioration of the cells at 32°C was observed and the number of cells was only 32% of the number at 28°C, which was still the best according to the counts of nuclei. Cells growing at 24°C were still in good condition but the

Fig. 13. Optimal osmotic pressure for the growth of AC20 in medium D. Values represented by ⏃ and ⏁ were obtained by applying the nuclear area correction and the averages of corrected values are represented by ⊗ points joined by the broken line.


Osmotic Pressure
Number of Cells As % of Maximum
110
100
90
80
70
60
50
40
310
320
330
340
350
360
370
380
390
400
milliosmols per kilogram of water
Optimal osmotic pressure
Exp1
Exp2
Corrected Number Exp1
Corrected Number Exp2
For 1 flask
Average

rate of growth at this temperature was only 82% of that at 28°C. AC20 cells have been subcultured at 4 to 5 day intervals more than 190 consecutive times at 28°C. Both AS and AN cells have been subcultured for more than 100 times at this temperature.

2. Short term cell storage at 13°C

Schlegel and Black (1969) demonstrated the possibility of storage of viable AC2 cells for long periods at 9°C. Liu (1969) was able to store AC cultures at 9°C for as long as 375 days, and he recovered AS cells after as long as 132 days of such storage. His AS cells did not store well at either 9 or 12°C. Using the data of Schlegel and Black (1969) on doubling of the population at different temperatures, it was estimated that storage at about 13°C would be better. A refrigerated incubator with 5 shelves was adjusted to give this temperature on the middle shelf. The temperature on the highest shelf was about 11°C and on the lowest one about 15°C. In this incubator it has been possible to store AC, AN and AS cell lines for long periods. Before moving cells to such storage, the culture medium was removed and replaced with 5 ml of fresh culture medium, medium that was replaced with 5 ml of fresh medium every 3 to 4 months. As described above in the study of the changes of pH of the medium during cell storage at 13°C, the changes in the pH of the medium during storage were greatly reduced after the medium was buffered with histidine. Recovery of cells after storage was also improved. It has been possible to store both healthy and WTV-infected AC20 cells in one flask without subculture for 21 months at 13°C and to recover viable cells. AN cells were stored for as long as 11 months and AS cells for as long as 14 months, and even though recovery of the cells has not yet been made after the longer storage periods they appear to be viable when

observed under the phase contrast microscope. Recovery of AS cells after storage at 13°C has been successful after periods of 6 to 8 months. This difference with earlier results (Liu, 1969) may be due to the improved conditions of the cells in the new media used. Cell monolayers that have been growing at about 28°C for 2 or 3 days and are almost confluent before storage at 13°C appear to be maintained better in storage than younger monolayers.

N. Enzymatic Detachment of the Cells

1. Trypsin

Trypsin 1:250 has been used since the beginning of work with insect tissue culture in this laboratory. A concentration of 0.25% trypsin in Rinaldini's salt solution was used by Chiu et al (1966) to dissociate cells from embryos of A. constricta. The concentration of trypsin was reduced to 0.05% in Rinaldini's salt solution at pH 7.5, for detachment of cells at the time of subculture (Chiu and Black, 1967). This trypsin treatment for periods of time between 4 and 10 minutes has been the principal method used in this laboratory, but because of difficulties observed in obtaining good dissociation of the cells after the use of medium buffered with histidine, and because this treatment was being made at pH 7.5, more than 1.0 pH unit away from the optimal (pH 6.43) for the growth of AC20 cells, and because aggregation of cells was a problem, several experiments were designed to try to improve cell detachment.

The possibility of using trypsin suspension at pH 6.4 was studied by preparing trypsin solutions at a concentration of 0.5, 0.1 and 0.05% trypsin in Rinaldini's salt solution and adjusting the pH to 6.4 with 1.0 N HCl before Millipore filtration. When these preparations were used to detach AC20 cells from monolayers in flasks, no detachment occurred. It was known that

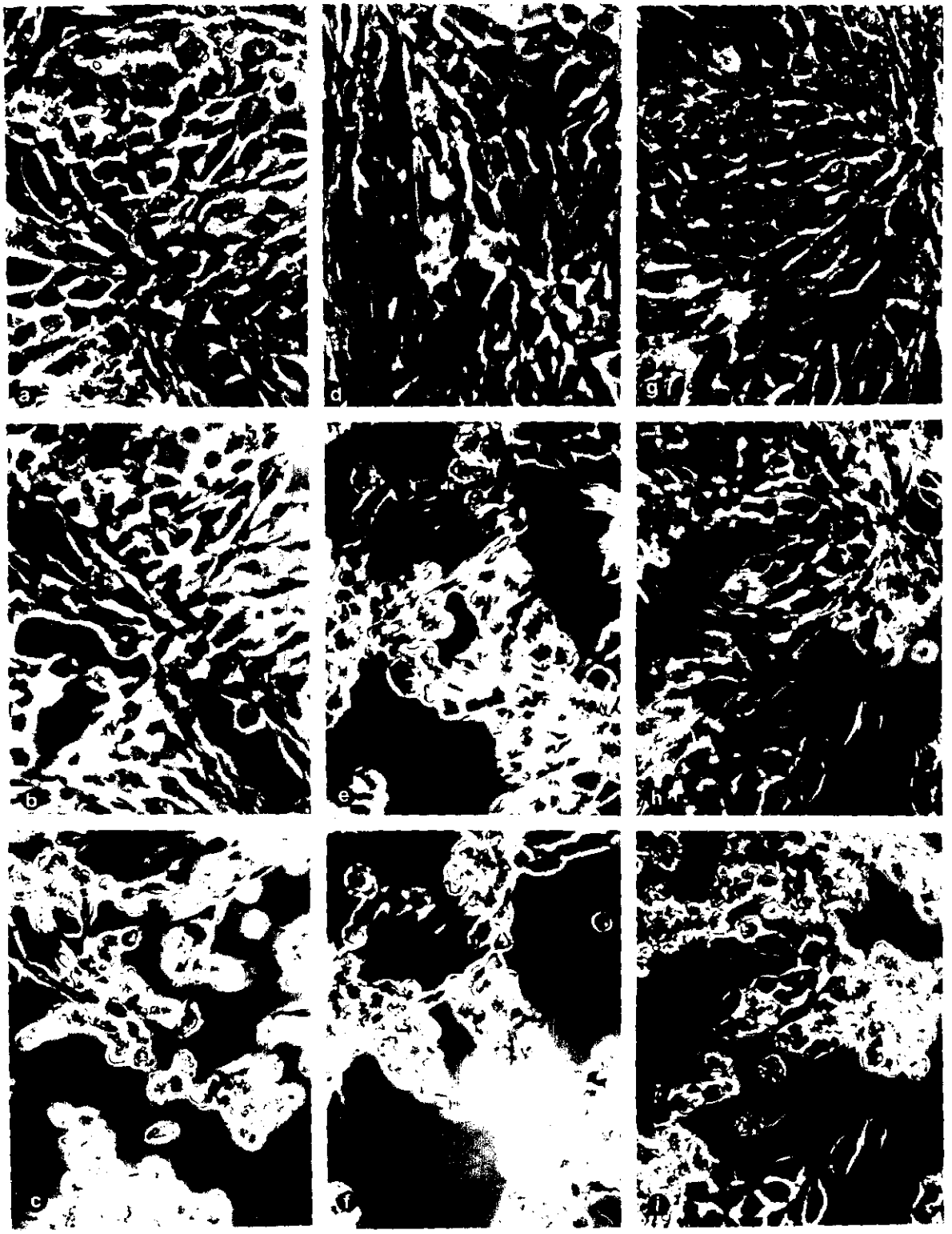
pH 6.4 was far from the optimal pH for the activity of trypsin. The results of this experiment, in which even high concentrations of trypsin failed to detach cells, led to the abandonment of attempts to use trypsin at the optimal pH for growth of AC20 cells.

Longer trypsin treatments at pH 7.5 and a higher concentration of trypsin were tested to determine if better dissociation of the cells would occur than with 0.05% trypsin at pH 7.5 for 4 to 10 minutes. Four different commercial samples of trypsin were prepared at 0.25% concentration in Rinaldini's salt solution at pH 7.5. When AC20 cells were treated with any one of these four preparations for 15 minutes and then subcultured, the number of cell aggregates had increased 24 hours later. This condition, which was not observed in the control treated for 4 minutes with 0.05% trypsin, has always been present when a treatment is detrimental to the cells. It was concluded that an improved method was not likely to be obtained by use of greater concentrations of trypsin or longer treatments.

2. Papain

Papain was studied because its optimal activity occurs at pH 6.4, the optimal pH for growth of AC20 cells. Papain was prepared in a solution of 0.02% Versene in normal saline (Flow Laboratories, Rockville, Maryland), at concentrations of 0.1, 0.05 and 0.01 mg of papain per ml. Each of these preparations was adjusted to pH 6.4 with a solution of 0.05 M cysteine, one of the activators of the enzyme (Kimmel and Smith, 1954). When these preparations were used to detach AC20 cells from the culture flask, it was observed that with all the treatments the cells started to dissociate as early as 2 minutes after the beginning of the treatment. The detachment of the cells was as efficient at 0.01 mg/ml as at 0.1 mg/ml (Fig. 14).

Fig. 14. Detachment of AC20 cells from the culture flask with different concentrations of papain. Figs. a, d and g: Cells before treatment. Figs. b and c: Cells in microscopic field "a" after treatment with 2 ml of a solution containing 0.01 mg papain/ml for 2 min (b) and 5 min (c). Figs. e and f: Cells in microscopic field "d" after treatment with a concentration of 0.05 mg papain/ml for 2 and 5 min, respectively. Figs. h and i: Cells in microscopic field "g" after treatment with a concentration of 0.1 mg papain/ml for 2 and 5 min, respectively. (Phase contrast microscopy, x255)


a
d
g
b
e
h
c
f
i

Two ml of a papain suspension of 0.01 mg/ml for 5 minutes was used in further tests. When longer periods of papain treatment at concentrations of 0.1, 0.05 and 0.01 mg/ml were tested, there was no adverse effect on the cells, and the enzyme was no longer active after 10-20 minutes.

Transferred cells started to attach to the flask faster after papain treatment than after the best trypsin treatment. In addition to the faster attachment, the appearance of cells was better, and there were fewer cell aggregates (Fig. 15). However, when the effect of papain treatment on the growth of AC20 cells was compared with the effect of trypsin treatment, under optimal conditions for both treatments, significant differences in the rate of growth were not detected.

Because the osmotic pressure of the solution used as diluent for the enzyme was 280 mOsm, an osmotic pressure lower than the optimal for growth of AC20 cells, a new diluent solution was prepared (Table 11). In this solution KCl and NaCl were included in twice the optimal concentrations used in medium D. K_2HPO_4 was used instead of KH_2PO_4 in order to increase the pH of the solution and the molar concentration was twice that of KH_2PO_4 in the medium. Disodium dihydrogen ethylenediaminotetraacetate dihydrate (TitraVer) (Hach Chemical Co., Ames, Iowa) was added to give a molar concentration of 0.001, the concentration used in the assay tests of papain, as one of the stabilizing agents (Kimmel and Smith, 1954). The pH of the solution was adjusted to 6.4 with 351 mg of cysteine per liter. This amount of cysteine was less than half the amount used in the assay tests of Kimmel and Smith (1954) who used 878 mg/liter (0.005 M) because higher amounts brought the pH below 6.4. Dextrose was added in the concentration required to provide a final osmotic pressure of 360 mOsm. Antibiotics were added in the same

Fig. 15. The effect on AC20 cells of treatments with 0.01 mg papain/ml of suitable diluent or 0.05% trypsin in Rinaldini's salt solution. Cells in "a" to "e" represent different microscopic fields in the culture treated with papain and cells in "f" to "j" represent different microscopic fields in the culture treated with trypsin. Figs. a and f: Cells before treatment. Fig. b: Cells after 5 min of treatment. Figs. c, d, and e: Cells after subculture for 1, 3 and 24 hours, respectively. Fig. g: Cells after 5 min of treatment. Figs. h, i and j: Cells after subculture for 1, 3 and 24 hours, respectively. (Phase contrast microscopy, x200)

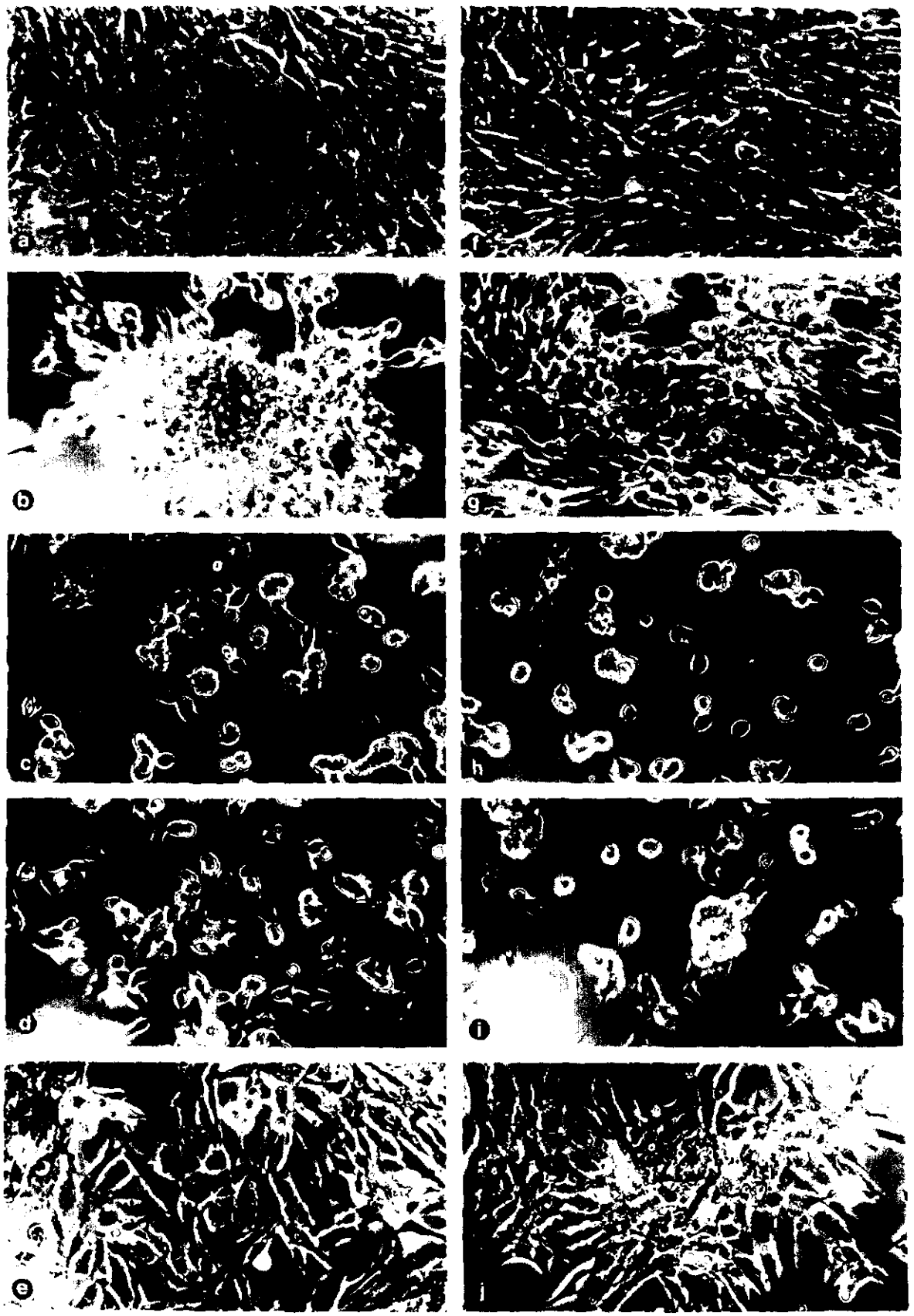

a
b
c
d
e
f
g
h
i
j

Table 11

COMPOSITION OF DILUENT SOLUTION FOR PAPAIN

KCl	3,200 mg/liter
NaCl	2,000 mg/liter
K_2HPO_4	690 mg/liter
Dextrose	33,000 mg/liter
TitraVer	380 mg/liter
Cysteine	351 mg/liter
Penicillin G	100,000 units
Streptomycin	100 mg/liter
Neomycin	50 mg/liter
pH	6.4
Osmotic pressure	360 mOsm

concentration used in preparation of the culture medium.

Two ml of this diluent solution was used to wash the cell monolayer after the growth medium had been removed. Then 2 ml of a solution of papain, 0.01 mg/ml, in the diluent solution was added to the monolayer. Enzymatic action was allowed to take place for 5 minutes, action that was stopped by adding 2 ml of fresh medium to the flask. Cells were removed from the flask and transferred to a conical centrifuge tube to follow the procedure described for subculturing in "Materials and Methods."

Both diluent and papain solutions were stored at 4°C. Twice crystallized papain from Nutritional Biochemicals Corporation, Cleveland, Ohio, was prepared as needed for one or two week periods by diluting their preparation in the diluent to give a concentration of 0.1 mg/ml. After passage through a 0.22 µm Millipore filter it was diluted to 0.01 mg/liter. Preparations of papain in diluent were still active after 57 days storage at 4°C.

O. Doubling Time

In an experiment on the doubling time of a cell population in medium B, measurements of nuclear diameters clearly demonstrated a progressive decrease in nuclear area with time after subculture (Table 12). The great majority of nuclei in these experiments had diameters varying from 5 to 7 units of length on an ocular micrometer scale. Each unit of length (u) (i.e. 1 scale division on the ocular micrometer) measured 1.88 µm, so that the nuclei varied in diameter from 9.4 to 13.2 µm. However, rather than measuring nuclear diameters in µm, it was actually easier and more efficient to determine an average nuclear diameter by recording the diameters of 10 individual nuclei in each flask to the closest integral number of u. Among the nuclei falling on the points of the reticule (Fig. 1) in each microscopic field,

Table 12

AREA CORRECTIONS OF THE AVERAGE NUMBER OF NUCLEI

Hours after subculture	Average number of nuclei per field	Average diameter (u)*	Square of average diameter			Average of squared diameters		
			Actual (u^2)	Ratio: 24 hr ÷ other time	Corrected number of nuclei per field	Actual (u^2)	Ratio: 24 hr ÷ other time	Corrected number of nuclei per field
24	4.10	7.59	57.60	1	4.10	59.24	1	4.10
44	4.70	7.25	52.70	0.91	5.16	54.05	0.91	5.16
56	5.40	7.32	53.58	0.93	5.81	54.28	0.92	5.87
68	6.60	6.11	37.33	0.65	10.15	38.52	0.65	10.15
80	7.30	6.13	37.57	0.65	11.23	38.68	0.65	11.23
92	7.40	5.85	34.22	0.59	12.54	34.90	0.59	12.54
104	8.40	5.65	31.92	0.55	15.27	32.57	0.55	15.27
117	9.70	5.54	30.69	0.53	18.30	31.66	0.53	18.30
146	9.60	5.27	27.77	0.48	20.00	28.80	0.49	19.59
170	10.85	5.24	27.45	0.48	22.60	28.20	0.48	22.60
192	11.20	5.34	28.51	0.49	22.86	29.02	0.49	22.86
214	11.60	5.44	29.59	0.51	22.74	30.00	0.51	22.74

*One unit of length (u), i.e., 1 scale division on an ocular micrometer, equaled 1.88 μm.

the one whose diameter was easiest to measure was selected for the purpose. The measurement of the 10 nuclei, 1 nucleus in each of 10 fields per flask, required about 10 minutes. On the assumption that the opportunity for any nucleus to coincide with a point was proportional to its area, all counts in the following experiment were corrected for nuclear areas. The average nuclear area was then calculated firstly by squaring the average diameter in a set, and secondly and more correctly, by averaging the squared diameters in a set. Both methods gave essentially the same corrected results (Table 12) and indicated that unless the variation in nuclear diameters is great enough to give significantly different results there is no need for the more laborious second method of calculating the average nuclear area (Table 12). Enumeration of the nuclei in a flask at frequent intervals without disturbing the cell monolayer showed that there were alternating periods of rapid and slow increase in numbers of nuclei (Table 12).

The average doubling time between 24 and 96 hours after subculture in medium B was about 45 hours (Table 13), a 25% reduction in the best doubling time (60 hours) in medium A (Martinez-Lopez, 1971).

The doubling time of AC20 cells growing in medium D was measured between 24 and 48 hours and between 24 and 96 hours after subculture. In one of four experiments (experiment 4) the counts for the 24 to 48 hour period were adjusted, using the square of the average diameter of the nuclei to provide the correction for nuclear area (Table 13). With this correction the average doubling time in experiment 4 between 24 and 48 hours was reduced from 60 hours to 49 hours. When the same correction factor was applied to the average doubling time of 39 hours for the four experiments, it was reduced to 25 hours for the 24 to 48 hour period. The average apparent doubling

Table 13

DOUBLING TIME OF AC20 CELL POPULATION

Experiment	For 24-48 hr	Nuclear area correction	For 24-96 hr	Nuclear area correction
		In medium B		
1	122 hr	No	84 hr	No
	72 hr	Yes	45 hr	Yes
		In medium D		
1	44 hr	No	41 hr	No
2	32 hr	No	66 hr	No
3	19 hr	No	38 hr	No
4	60 hr	No	57 hr	No
	49 hr	Yes		
Average (medium D)	39 hr	No	51 hr	No
	25 hr	Yes		

time in medium D between 24 and 96 hours after subculture was 51 hours (Table 13). By mistake, no nuclear area correction was measured for this period in medium D.

As the original medium was being modified in accord with determinations of optimal concentrations, a shorter doubling time was observed both in the second 24 hours of a 4-day growth period and in the whole 4-day period. Cells in mitosis were noticeably more common. Moreover, the cells had an improved appearance, cell debris was greatly reduced or eliminated and it was possible to go from a regimen of 1 to 2 subcultures every 4 to 5 days to a regimen of subcultures of 1 to 3 every 3 or 4 days and as high as 1 to 6 every week. In some experiments in which a large number of cells were required, the possibility of making 1 to 3 or 1 to 4 subcultures was very useful. This was possible because the improved medium and the improved cell detachment procedure permitted lower seeding densities at the time of subculture.

P. Preparation of Medium D.

Once the optimal concentration of the components in the medium was determined, and the optimal osmotic pressure was studied, the best sequence for adding the medium components was studied in order to reduce the formation of irreversible precipitates. Different combinations of the salts used in medium D were mixed at 3x the concentration in medium D. In these experiments it was observed that an irreversible precipitate formed when $NaHCO_3$ and $CaCl_2.2H_2O$ were mixed at this concentration. The precipitate formed faster when $NaHCO_3$ was added as a powder to the $CaCl_2.2H_2O$ solution and was delayed when each one of the salts was dissolved first in half the final volume. There were no precipitations with any combinations of the other salts. For these reasons the $NaHCO_3$ and histidines were first dissolved separately in somewhat less than

half the total volume of water. The water used in all medium preparations was glass distilled water with a specific conductance of 1.25 micromhos when free of CO_2 and measured at 25°C.

It was found convenient to prepare batches of 4.5 liters of medium at a time. An antibiotic mixture was prepared and stored frozen at -25°C in 50 ml aliquots so that each aliquot could provide the quantities needed for 4.5 liters. The FBS was heat-treated as described in "Materials and Methods."

To prepare 4.5 liters, the histidine-HCl, histidine (free base) and the $NaHCO_3$ were dissolved in that order in 2 liters of water to form solution A. It should be emphasized that the proportions of histidine in Table 1 need to be varied somewhat according to the different buffering capacities of different batches of some of the components. $CaCl_2$, $MgSO_4$, KH_2PO_4, KCl, NaCl, dextrose, antibiotics, Yeastolate, the heat-treated FBS and lactalbumin hydrolysate were added in that order with stirring to a vessel containing about 1,100 ml of water to form solution B. Solutions A and B were mixed, brought to 4.5 liters and sterilized by filtering through 0.8, 0.45 and 0.22 μm Millipore filters as described in "Materials and Methods."

Basic medium D is readily adjustable in certain ways to make it more suitable for growth of certain cell lines other than AC20. For example, the amounts of histidines were varied to obtain medium D with a pH of 6.30 which is optimal for the growth of AS2 cells. It is possible that experiments with still other cell lines will reveal other preferred pH values for medium D. Other cell lines may prefer a different osmotic pressure than AC20; the experiments on the osmolarity of medium D show that it can be readily altered. Medium D can be used with a variety of antibiotics and gentamicin alone may

prove to be adequate. Perhaps it is worth noting here that some of the antibiotics used in medium D, antibiotics that seem not to interfere with virus inoculation of the insect cells, may have to be omitted in preparations of medium to be used in studies of mycoplasma- or rickettsia-like plant pathogens.

Effect of Improved Media on Virus Infection of Cells

A. Susceptibility to Wound Tumor Virus Inoculation

AC20 cell monolayers that had been growing on either medium B or medium D were subcultured in the corresponding medium in glass coverslips prepared as described in "Materials and Methods." The monolayers obtained were inoculated 24 hours after subculture with a $10^{-4.5}$ dilution of the purified preparation of WTV VI72 provided by Dr. D. V. R. Reddy. Following the procedure described by Kimura and Black (1971), the monolayers were washed twice with histidine-$MgCl_2$ (His-$MgCl_2$) buffer at pH 6.0, inoculated with 0.1 ml of inoculum and incubated for 2 hours at 28°C to accomplish inoculation. At the end of the inoculation period, the inoculum was removed, the monolayers washed twice with growth medium, and then each coverslip was covered with 0.1 ml of corresponding fresh growth medium. Inoculated monolayers were then incubated at about 28°C for 48 hours. After the incubation period, cell monolayers were fixed and stained with WTV fluorescent antibodies following the procedures as described by Chiu and Black (1969), Liu (1969) and Kimura and Black (1971). An index value of the proportion of infected cells was determined for each medium tested, following the indexing procedure described in "Materials and Methods." The susceptibility of AC20 cells in medium B to WTV infection was only 62% of the susceptibility of the cells in medium D (Table 14).

Table 14

SUSCEPTIBILITY OF CELLS IN MEDIA B AND D

TO VIRUS INOCULATION

Coverslip	Cell line	Virus	Medium B			Medium D		
			a/b*	ratio	average	a/b*	ratio	average
1	AC20	WTV	33/61	0.54		58/67	0.87	
			35/63	0.55	0.52	61/71	0.86	0.84
2	AC20	WTV	35/66	0.53	(62%)**	59/73	0.81	(100%)**
			31/63	0.49		66/81	0.82	
1	AC20	CYDV	12/59	0.21		23/70	0.33	
			16/62	0.26	0.21	25/89	0.28	0.33
2	AC20	CYDV	13/61	0.21	(62%)**	24/79	0.30	(100%)**
			12/61	0.19		29/88	0.33	
1	AS2	SYDV	33/81	0.41		51/118	0.45	
			29/73	0.39	0.39	58/106	0.55	0.49
2	AS2	SYDV	44/104	0.42	(79%)**	57/114	0.50	(100%)**
			37/110	0.33		52/112	0.45	

*a/b a: average number of infected cells per microscopic field.

b: average number of cells in the central square of the reticule.

**Percentage of best infection in a given virus test.

B. Susceptibility to Potato Yellow Dwarf Virus Inoculation

1. Constricta Yellow Dwarf Virus

AC20 cell monolayers that had been growing in either medium B or medium D were subcultured in the corresponding medium on glass coverslips. The monolayers obtained were inoculated 24 hours after subculture with a $10^{-3.0}$ dilution of a purified preparation of CYDV as defined by Hsu and Black (1973b) and provided by Dr. H. T. Hsu. Following the procedure described by Hsu and Black (1973b), the monolayers were washed twice with histidine-magnesium chloride buffer at pH 5.26, inoculated with 0.1 ml of inoculum and incubated for one hour at about 28°C to accomplish inoculation. At the end of the inoculation period, the inoculum was removed, the monolayers washed twice with growth medium, and then each coverslip was covered with 0.1 ml of the corresponding fresh growth medium. Inoculated monolayers were then incubated at about 28°C for 48 hours. After this incubation period, cell monolayers were fixed and stained with CYDV fluorescent antibodies. The proportion of infected cells was determined as described in "Materials and Methods." The results (Table 14) of inoculation of AC20 cells with CYDV indicated that the susceptibility of cells in medium B was 62% of the susceptibility of cells in medium D.

2. Sanguinolenta Yellow Dwarf Virus

AS2 cell monolayers that had been growing in either medium B or medium D (pH 6.30) were subcultured in the corresponding medium on glass coverslips. The monolayers obtained were inoculated 24 hours after subculture with a $10^{-3.0}$ dilution of a purified preparation of SYDV as defined by Hsu and Black (1973b) and provided by Dr. H. T. Hsu. Following the procedure described by Hsu and Black (1973b), the monolayers were washed twice with histidine-magnesium chloride buffer at pH 6.00, inoculated with 0.1 ml of inoculum and

incubated for one hour at about 28°C to accomplish inoculation. At the end of the inoculation period, the inoculum was removed, the monolayer washed twice with growth medium, and then each coverslip was covered with 0.1 ml of the corresponding fresh growth medium. Inoculated monolayers were fixed and stained with SYDV fluorescent antibodies. The proportion of infected cells, determined as described in "Materials and Methods," showed that the susceptibility of AS2 cells to SYDV in medium B was 79% of their susceptibility in medium D (Table 14).

DISCUSSION

It has been possible to improve conditions for successful culture of AC20 cells derived from the leafhopper A. constricta. Less extensive experiments indicate that conditions for growing cell lines from other agallian leafhoppers have also been improved. The improved conditions will probably increase opportunities for establishing cell lines of other leafhopper vectors of plant viruses or mycoplasma- and rickettsia-like plant pathogens that are transmitted by leafhoppers.

This improvement of a medium, thus far the only one with which successful cell lines from leafhoppers have been established (Chiu and Black, 1967; Richardson and Jensen, 1971; Windsor, 1972) was favored by the availability of an established cell line that provided large numbers of cells. It was also facilitated by the development of the counting technique with the phase contrast microscope. With this procedure for indexing changes in the number of cells in cultures, it was possible to reduce the number of flasks required per treatment from about 20 (Martinez-Lopez and Black, 1971) to 2.

At the same time, the ease of replication of experiments and the precision of the results was increased. This was attained partly by avoidance of errors introduced during harvest and dilution of the cell preparations, partly by reduction of difficulties experienced during detachment and dissociation of the cells, for example, the formation of cell aggregates and the production of debris, which may affect counts made by the Coulter Counter.

Fixed positions on the mechanical stage for the fields to be counted made it possible to make counts more rapidly than would be the case if the

positions were picked at random for each flask examined. The results with ten fixed positions were satisfactory. Where distribution of the cells in the flask is less uniform, it might be necessary to select the field positions at random and increase the number of fields counted. This could easily be done, but it would make the process of counting slower. In general, counting without correction for variations in nuclear areas provided adequate information about differences between treatments. However, wherever treatments appreciably affect the area of the nuclei, it is necessary to use measures of relative nuclear area to obtain more precise information. It was assumed that in comparisons between several concentrations of a medium component there was no necessity to apply the nuclear area correction; but on the other hand, in experiments such as those on doubling time, or osmotic pressure, correction for the nuclear area was essential.

The data presented on the sensitivity of the technique and the comparison between pairs demonstrated that the members of a duplicate pair differed little enough so that they made such a pair a satisfactory treatment unit. With any other technique tried in this laboratory, it was never possible to get such good agreement between members of a pair. Because the cells of the monolayer were not damaged or disturbed by the counting procedure, it was possible to obtain much more information from each flask than with counting methods that required removal of the cells. It was possible to make counts as desired on any flask without introducing variations, some of which were made worse when large numbers of replicate flasks were required, as in all the techniques in which harvesting of the cells occurred.

The standard counting procedure with the phase contrast microscope sampled 40 microscopic fields, 10 at each of 4 different times, in each flask.

In the average experiment with 10 flasks, 400 samples are counted. Generally, methods which enumerate all the cells in a microscopic field directly under the microscope or in a photograph use many fewer samples. For these reasons the counting method with the phase contrast microscope appears to provide less variability between replicates and more precision.

The direct enumeration of cell nuclei by phase contrast microscopy, besides providing the investigator with quantitative information about the growth of the cell monolayer, coincidentally resulted in observations on cell appearance, proportion of mitotic cells and cytologic changes that provided indications about the condition of the cells.

The observation, during some experiments and especially during the doubling time experiments, that there were alternating periods of rapid and slow increase in the number of nuclei, suggested that more than one count during the growth period being studied was necessary to avoid certain misinterpretations of results. This is one more advantage of a counting technique that does not disturb the cell monolayer. The average of four counts at 24 hour intervals was very convenient.

The resolution at high magnification now available in the phase contrast microscope with a working distance of 4 cm between phase contrast disc and the object, has been one of the most important factors in the utilization of this procedure. When lower magnifications were tried, it became more difficult and less accurate to enumerate the cell nuclei that coincided with the reticule points. This high magnification was also a requirement when the nuclear diameters had to be measured. The optical properties of the plastic flasks were superior to those of glass vessels tested. Although the counting technique was developed for measuring growth in monolayers of cells of the

insect Agallia constricta, a vector of WTV and PYDV. However, it should be applicable to other cell types growing as monolayers in containers having optical properties satisfactory for observation of cells by phase microscopy.

An estimate of the absolute number of cells in a flask can be obtained from calculations based on the area of the bottom of the flask (25 cm^2) and average value for the square of the diameter of the nucleus in μm^2, by relating average counts of nuclei on the reticule points (corrected for nuclear area) and average counts of cells in a measured small area of the microscopic field. This may be illustrated by the following specific example which can be taken as a standard reference. A circular glass plate mounted with the ocular lens was marked to delimit a small square area in the microscopic field with the lens system used in this study which gave a total magnification of 500x. This square measured 77 µm on a side and contained 5.93×10^{-5} sq. cm. An average flask monolayer, based on counts on 10 flasks, had an average of 6.28 cells in this area and would contain $\frac{6.2 \times 25}{5.93 \times 10^{-5}}$ or 2.65×10^6 cells. The average diameter of the nuclei was 11.73 µm and the average number of nuclei falling on the reticule points was 4.19. Any count of nuclei on the reticule points for a flask with an unknown cell population can be converted to total cells per flask by applying the data from the above selected reference in the following calculation:

$$\text{cells/flask} = 2.65 \times 10^6 \times \frac{(11.73)^2}{4.19} \times \frac{A}{B} \quad \text{or} \quad 86.9 \times 10^6 \times \frac{A}{B},$$

where A is the number of nuclei counted on points for the unknown and B is the square of the average diameter in µm of nuclei of the unknown. Provided the equipment used, and the ratio of nuclei to cells, is the same, the formula can be applied independently of species and conditions of culture without corrections.

The differences observed in suitability of different batches of FBS as a component in the insect tissue culture medium were comparable with the

results of Boone et al (1972) when working with human fetal lung cells. The counting technique with the phase contrast microscope made it easier to screen samples of FBS for the selection of the most suitable batch.

The studies on the optimal pH of the medium and the use of histidine as a buffer and as a nutrient indicated that the cells were very sensitive to small divergences of pH from the optimum. When the pH of the medium was not optimal, a change toward the optimum occurred. These changes apparently reveal an ability of the cells to adjust the pH of the medium towards the optimum for cell growth. This property of cells may make it desirable not to use a buffered medium with primary cultures of cells whose optimum pH is not known. A buffered medium may be expected to interfere with an action by the cells that would make the medium more favorable for their growth.

Changes in the pH of the medium have been observed in mammalian cell cultures (Phillips and Feldhaus, 1956; Eagle, 1971). Phillips and Feldhaus considered that this was because of a tendency of the pH of the medium to rise under acid conditions and to fall under alkaline conditions, so that the medium tended to change towards neutrality. If the optimal pH for the growth of the L cells used in their experiments was between 7.2 and 7.4 (Paul, 1959), it seems possible that the changes in the pH of the medium were of the same kind as those observed for medium sustaining the growth of AC20 cells. Eagle (1971) working with normal, virus-transformed and cancer cells with optimal pHs at points between 6.9 and 7.8, observed that in media buffered with $NaHCO_3$, there was a rise in pH due to early loss of CO_2. The rise was followed by acidification as a result of cell metabolism so that 24 hours later the pH was as low as 6.9 in cultures with dense cell populations. Here again it seems possible that the shift in the pH of the medium was towards the optimum.

Changes in pH of tissue culture medium have been explained by Zwarton and Westwood (1958). They showed that under more alkaline conditions more glucose was converted to lactic acid and more pyruvic acid was produced. Under more acid conditions on the other hand, lactic acid tended to disappear from the medium.

Histidine proved to be an excellent buffer for the leafhopper tissue culture medium at concentrations as low as 0.005 M. However, the beneficial effect of histidine at concentrations as high as 0.045 M above the unknown quantity of histidine already present in the medium from lactalbumin hydrolysate and Yeastolate, indicated the importance of histidine as a nutrient for the leafhopper cells.

Histidine has not been included in some of the studies of biological buffers (Good et al, 1966; Eagle, 1971), but its role in the buffer system of insect hemolymph was indicated by Wyatt (1961). Kimura and Black (1971) used histidine as a buffer in inoculations of insect cell monolayers with WTV. In some cases histidine accounts for up to 35% of the free amino acids in the hemolymph (Wigglesworth, 1965). Histidine has been used in insect tissue culture media at concentrations of 1,000 mg/liter (Vago, 1959), 2,500 mg/liter (Wyatt, 1956), but the results of the present study indicated that it was beneficial in the case of AC20 cells in concentrations of 8,300 mg/liter.

Increased amounts of dextrose provided better growth, and this sugar was used in the adjustment of the osmotic pressure of the medium to the optimum for the growth of the leafhopper cells. In the studies of osmotic pressure it was necessary to correct the counts made with the reticule by the square of the average diameter to prevent misleading results due to the

differences in the size of the nuclei at the different osmotic pressures tested. The use of trehalose, the major sugar of various insects (Wyatt, 1961), did not provide better growth.

The differences in the suitability observed in different batches of some of the components for the leafhopper tissue culture medium indicate that a single batch of any one component that is toxic can make the entire medium very damaging to a cell culture.

Studies on the optimal temperature for growth of AC20 cells indicated once more the harmful effect of temperatures of 32 or 36°C. The best growth in medium D was obtained at 28°C. The rate of growth of the cells was lower at 24°C, but no damage was inflicted on the cells. The optimal temperature is the same as that obtained by Schlegel and Black for AC2 cells (Black, 1969) in medium O, and Martinez-Lopez and Black (1971) for AC20 cells in medium A.

When the use of trypsin for the detachment of the cells from the culture flasks was studied it was observed that concentrations of 0.25% were harmful for the cells. This harmful effect had been previously observed by Day and Grace (1959), Hirumi and Maramorosch (1964), but with lower concentrations and shorter treatments this enzyme has been the one most widely used with insect tissue cultures (Martignoni et al, 1958; Grace, 1962; Chiu and Black, 1967; Schneider, 1969; Yamada et al, 1971; Vaughn, 1971). In the case of leafhopper cells the trypsin digestion has to be made at a pH more than 1.0 pH unit higher than the optimum for growth of the cells. In order to avoid this problem and some of the difficulties in dissociation of the cells and the formation of cell aggregates papain was tested. Although this enzyme was used by Rinaldini (1958) in some preliminary studies with mammalian cells

apparently it has not been used in insect tissue culture.

The mild action of papain, an enzyme that can be used at the optimal pH for the growth of most insect cell lines, and the efficient dissociation and detachment of the cells with treatment at concentrations of 0.01 mg papain per ml may make it very useful in work with insect tissue cultures. It may be especially useful in the dissociation of embryonic tissue during the initial steps in the establishment of a cell line.

The increased susceptibility of leafhopper cells to inoculation with either WTV, CYDV or SYDV viruses indicated once more the importance of vigorous cells for infectivity studies, and this indicated the possibility of increasing even further the sensitivity of the insect tissue culture technique for the study of plant viruses that multiply in insect vectors.

The improved medium obtained from these studies with A. constricta cells has already been useful in growing A. sanguinolenta and A. novella cells in culture. It seems probable that with small modifications medium D could produce even better growth of cells of these species of leafhoppers. The success of the medium with these other species suggests the possibility of using this insect tissue culture medium to obtain cell lines from other leafhoppers and possibly from other insects.

The improved medium, medium D, may be useful in studies on the preparation of a chemically defined medium for growth of agallian cells in tissue culture. Chemical analysis of medium D would provide information on a mixture of amino-acids, vitamins, nucleic acids, certain intermediary metabolites and accessory growth factors, which give satisfactory growth of AC20 cells. Some analyses on the composition of lactalbumin hydrolysate and yeast hydrolysate are available from commercial companies.

SUMMARY

Some of the optimal physical and nutritional conditions for tissue culture of cells of the insect Agallia constricta, a vector of certain plant viruses, were studied. A procedure was developed for the accurate, quick and simple indexing of the growth of cell monolayers by counting nuclei under reticule points in the eyepiece of an inverted phase contrast microscope at a magnification of 500x. For certain experiments it was necessary to obtain a measure of the area of the nuclei being counted, and to correct nuclear counts according to the relative areas of nuclei being compared, for example, at different times after subculture or in media of different osmotic pressure. This technique permitted handling large numbers of treatments simultaneously because of the small number of replicates necessary, and the small amount of time required to measure growth. On undisturbed cell monolayers, the technique made more information available than did other standard counting procedures, such as counts by hemocytometer or electronic cell counter.

Differences were observed in the suitability of batches of fetal bovine serum, lactalbumin hydrolysate and histidine as components in the insect tissue culture medium. These differences were demonstrated in samples from different commercial suppliers and in samples from a single supplier.

Optimal concentrations of salts, dextrose, lactalbumin hydrolysate, yeast hydrolysate, fetal bovine serum, histidine and antibiotics were determined. Fungizone (amphotericin B) at 50 or 100 mg per liter caused harmful effects. Optimal growth of A. constricta cells occurred at about pH 6.43. This pH was also close to the optimal pH for Agalliopsis novella cells.

Optimal growth for Aceratagallia sanguinolenta was at about pH 6.3. The pH of the medium remained unchanged during storage at about 4°C. Combinations of histidine-HCl and histidine (free base) made it possible to prepare buffered medium at any pH between 6.0 and 7.0. Optimal osmotic pressure was measured in different media at 310, 344 and 360 mOsm. Optimal temperature was about 28°C. A. constricta cells died in less than 24 hours at 36°C, and some damage to the cells was observed at 32°C. At 24°C growth of the cells was about 20% less than at the optimal temperature. Short term storage of the cells at 13°C with changes of medium every 3 to 4 months was very convenient. A. constricta cells were recovered after 21 months storage at this temperature.

Doubling time of A. constricta cells was reduced from about 74 hours in medium O, to about 60 hours in medium A, about 45 hours in medium B. The doubling time in medium D between 24 and 48 hours after subculture was about 23 hours. Insect cells subcultured in the improved medium had a better appearance as well as a shorter doubling time. The susceptibility of cells to wound tumor virus and to potato yellow dwarf virus was greater when they were grown in the improved medium.

Papain at 0.01 mg/ml was an excellent enzyme for detachment of the cells at time of subculture, perhaps because its optimal activity at pH 6.4 is the same as the optimal pH for growth of the cells.

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