Evaluation of Parameters in the Isolation of Viable Protoplasts from Cultured Tobacco Cells[1]

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ABSTRACT

A systematic evaluation disclosed the following conditions to be optimum for the isolation of viable protoplasts from cultured cells of *Nicotiana tabacum* L. 'Bright Yellow' grown in liquid suspensions: (a) the cell culture in the early phase of cell number increase, (b) an enzyme mixture of 1% cellulase "Onozuka" and 0.2% Macerozyme, (c) an enzyme solution pH of 4.7 or 5.7, (d) a 2- to 3-hr incubation period, (e) 5 ml of enzyme solution per 500 mg cells and contained in a 50-ml Delong flask, (f) agitation on a gyrotory shaker at 50 rpm, and (g) 0.3 to 0.8 M mannitol as osmoticum in the cell enzyme mixture. The incubation temperature may be varied from 22 to 37 C. The procedure enabled 30% of the tobacco cells to form protoplasts, 80% of which regenerated cell walls in 4 days and 40% resumed cell division activity when returned to cell culture medium.

There has been growing interest among diverse biologists in the experimental and practical applications of plant protoplasts. One of the most exciting possibilities with protoplasts is their use in the genetic transformation of higher plants and in their application to plant hybridization and breeding. Interspecific hybrid plants have already been obtained in one genus, *Nicotiana*, following fusion of somatic cell protoplasts (2). Somatic hybrid cells have been obtained by fusing protoplasts of several plant species as well as genera, and it remains now only to achieve the reconstitution of plants from such cells (14). The incorporation of foreign DNA into plant protoplasts has also been demonstrated (12). It is not unreasonable to expect that ultimately specific fractions of the DNA, perhaps even cistron units, will be employed to transform plants genetically using protoplasts.

Viable protoplasts have already been isolated from numerous plant species and various plant parts (1, 3, 4, 6, 7, 10, 11, 13, 15, 17, 18). While there have been some common features in the procedures of diverse authors, the detailed steps or conditions prescribed for each plant or plant part have been sufficiently different to suggest that perhaps specific conditions were required of each plant or plant material being processed. This research has attempted to assess systematically the relative importance of the factors which have been prescribed most frequently as significant in the isolation of plant protoplasts. It is hoped that this report will serve as a guide to the

parameters which require evaluation in the development of procedures for the isolation of viable protoplasts of any desired plant, tissue, or cells.

MATERIALS AND METHODS

Source of Protoplasts. The protoplast isolation experiments were conducted with cells of *Nicotiana tabacum* L. 'Bright Yellow' grown in liquid suspension culture. The suspension cultures were obtained from callus established on nutrient agar. The callus originated in stem sections and was maintained in stock by monthly subculture on an agar medium, the composition of which is shown in Table I under "Callus and Cell Culture." To obtain cells in suspension culture, 1 g of callus was inoculated into 25 ml of nutrient medium contained in 125-ml Delong flasks. The composition of the medium was the same as that of Table I, but without the agar. Whereas stock cultures of the callus were maintained in constant darkness, liquid suspension cultures were exposed 16 hr daily to 1000 lux Gro Lux light. The multiplication of cells was observed to be stimulated by the low intensity illumination. The temperature for both types of cultures was a constant 27 C. The liquid suspension cultures were agitated continuously at 150 rpm, using a New Brunswick Model G-10 gyrotory shaker. Each culture of suspended cells was recultured at least twice before they were used as source of protoplasts, each reculture passage lasting two weeks.

Isolation of Protoplasts. Initially, the reference or standard procedure of isolating protoplasts was as follows. Cell samples were obtained by centrifuging liquid suspension cultures at 300g for 10 min. Pellet portions of 500 mg of cells and cell aggregates were then resuspended in 5 ml of enzyme solution, contained in 50-ml Delong flasks. Cellulase Onozuka R-10 and Macerozyme R-10 were used as enzyme preparations. Both were obtained from Calbiochem, San Diego, Calif. The pH of the enzyme solutions was set at 5.7, using 0.1 N HCl or NaOH; special buffering agents were not used. The cell enzyme mixture was agitated on a gyrotory shaker at 50 to 100 rpm for 3 to 4 hr at 27 C. After incubation, the unaffected cell aggregates were removed by filtration through nylon cloth, the mesh size of which ranged from 150 to 200 μm. The protoplasts passed through the filter, whereas the cell aggregates did not.

Protoplast Isolation Efficiency. The number of cells contained in 500 mg of tobacco tissue was first determined. Samples of the pellet fraction, following centrifugation of the suspension cultures, were placed in 2.5 ml of 5% chromium trioxide solution and agitated at 100 rpm for 24 hr. The temperature during agitation was 27 C. The incubated mixture was diluted to 25 ml with tap water, and the cell concentration in 0.1 ml aliquots was determined by the method of Henshaw *et al.* (5). The number of protoplasts in the filtrate fraction was

Table I. *Nutrient Medium Composition for Tobacco Tissue and Protoplast Cultures*

pH was adjusted to 5.7 with 0.1 N NaOH.

Constituent	Callus and Cell Culture	Protoplast Culture
	mg/l	
Mineral salts	—[1]	—[1]
Organic substances		
2,4-D	2	
NAA		0.6
Kinetin	0.25	0.1
Thiamine·HCl	10	10
Nicotinic acid	5	5
Pyridoxine·HCl	10	10
Myoinositol	100	100
Glycine	2	2
Sucrose	30,000	15,000
D-Mannitol		97,500
Complex addenda		
Casein hydrolysate[2]	2,000	
Difco Bacto-agar[3]	8,000	

[1] Murashige and Skoog formulation.

[2] Enzymatic digest from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] For cell suspension cultures agar was omitted.

determined next. Protoplast isolation efficiency was calculated as a ratio of the number of protoplasts isolated to the number of cells in the 500-mg tissue sample. Multiplication of this ratio by 100 gave per cent protoplasts.

Viability of Protoplasts. The viability of the isolated protoplasts was established by observing their behavior upon further cultures *in vitro* under aseptic conditions. The protoplast fraction was refiltered through three layers of cheesecloth and centrifuged at 100g for 2 min. The pellet was resuspended in 0.7 M mannitol solution and recentrifuged. This process of washing with mannitol solution was repeated three times. The washed protoplasts were resuspended in 0.7 M mannitol and 0.5 ml of the protoplast suspension was transferred to 4.5 ml of nutrient medium contained in 50-ml Delong flasks. The nutrient medium was of the same composition as shown under "Protoplast Culture" in Table I. The protoplast density was kept at 10^5 to 10^6 per ml. Cultures were maintained at constant 27 C and under 16 hr daily exposure to 1000 lux Gro Lux light. The regeneration of cell wall was observed on samples that had been stained with Calcofluor White ST for fluorescence microscopy (American Cyanamid Co., Wayne, N. J.), following the method of Nagata and Takebe (11).

RESULTS

The experiments were carried out in a sequence following the order in which the results are presented below. During the course of the investigation, the conditions of successive experiments were modified by incorporating any changes that were appropriate.

Growth Phase of Cell Suspension Culture. The isolation of protoplasts from cells grown in liquid suspension cultures was influenced by the growth phase the cell culture was in at the time samples were obtained. The data showing relationships between characteristics of the cell cultures and those of the protoplasts isolated from them can be seen in Figures 1 through 3. Samples of cell suspension were obtained at 2-day intervals and their fresh weight, cell concentration, and yield of protoplasts were determined. The protoplasts were obtained by

employing a solution containing 3% cellulase Onozuka, 0.2% Macerozym, and 0.7 M mannitol.

As is evident in Figure 1a, there was no change in the total fresh weight of cells in suspension cultures during the first 4 to
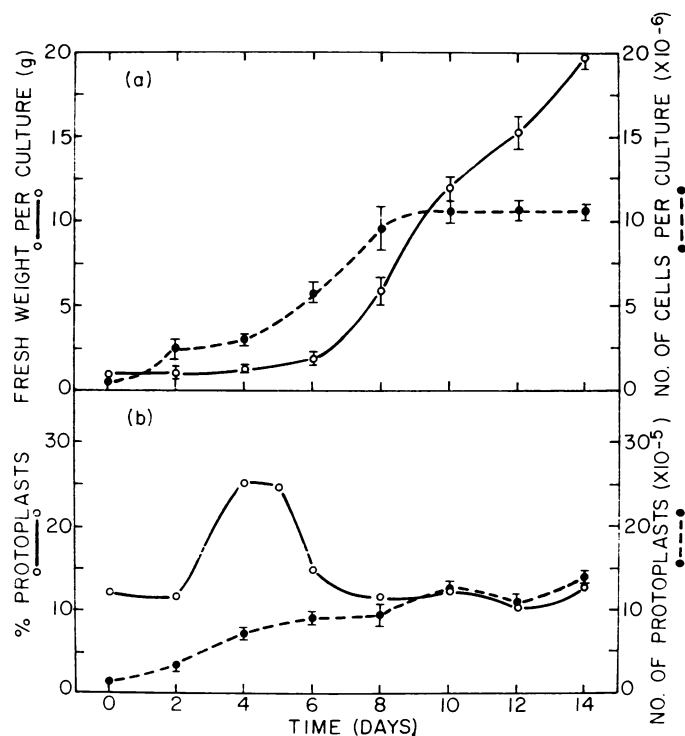

FIG. 1. Relationship between growth phase of tobacco cells in suspension culture and isolable protoplasts. a: Growth of cell cultures as reflected by fresh weight and cell number alterations; b: protoplasts isolated at various times during development of the cell culture. Incubation mixture contained 3% cellulase Onozuka, 0.2% Macerozyme, and 0.7 M mannitol.
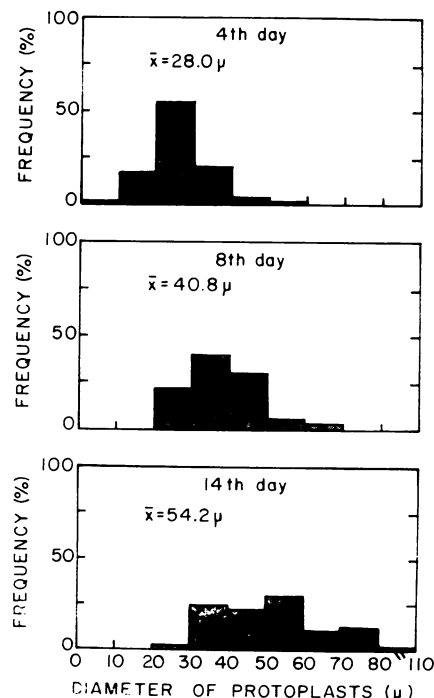

FIG. 2. Frequency distribution of protoplasts of various diameters isolated from tobacco cell cultures at 4, 8, and 14 days in passage.
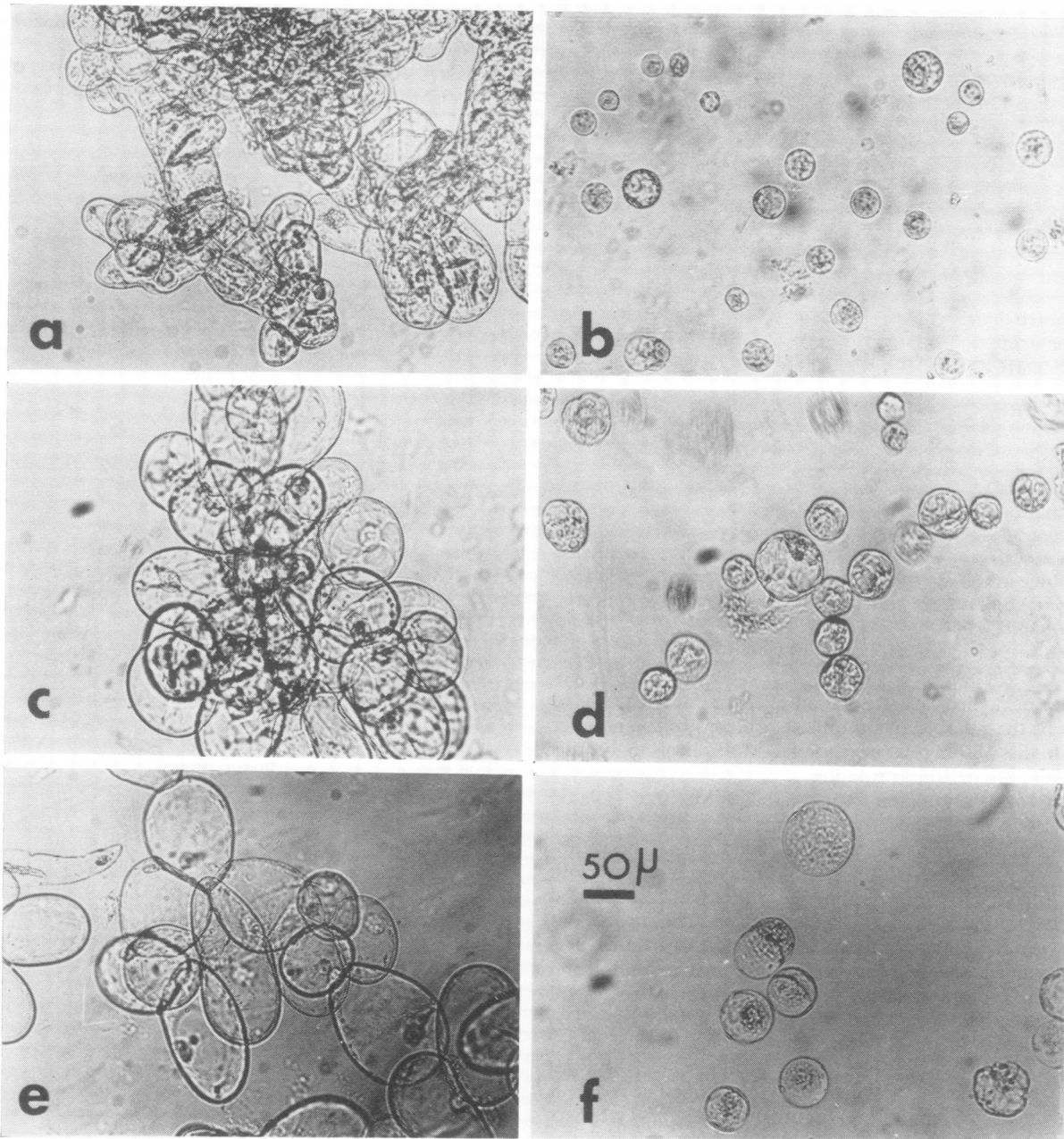
FIG. 3. Cells and corresponding tobacco protoplasts from: a,b: 4-day-old cell culture; c,d: 8-day-old cell culture; e,f: 14-day-old cell culture. Cells are on the left and protoplasts are on the right.

5 days. Subsequently, and beginning about the 6th day, there occurred a logarithmic rise in the fresh weight of cells; this rise continued until the end of the experiment, or the 14th day. The number of cells in the same suspension, however, showed immediate increase. This increase of cell concentration appeared to take on an exponential characteristic on the 4th or 5th day and remained so until the 8th day. No increase in number of cells occurred after 10 days. The increase of fresh weight after 10 days, therefore, was entirely due to an increase of cell size and not to cell division.

The number of protoplasts isolated from the above cells increased progressively throughout the experiment, although only very gradually. The efficiency in yield of protoplasts, expressed as per cent of protoplasts obtained in a given sample of cell suspension, was noticeably higher in samples obtained on the 4th and 5th days (Fig. 1b). Whereas all other samples of

cell cultures produced 12 to 15% protoplasts, those of the 4- and 5-day-old cultures gave about 25% protoplasts, or twice as much.

The size of protoplasts was directly related to the age of the cell culture from which the protoplasts were obtained. As is evident in Figure 2, protoplasts isolated from 4-day-old cell suspension cultures were about 28 μm in diameter, whereas those from 8-day-old cultures were 41 μm, and those from 14-day-old cultures were about 54 μm. This difference in size of protoplasts could be attributed to the variation in size of cells and particularly the degree of vacuolation of the cells. Figure 3, a to f, show the appearance of the cells and of their protoplasts in samples obtained at the above ages of cell suspension cultures. As these figures show, there was clearly a difference in protoplast size among the samples. Furthermore, there was an increase in the extent of vacuolation in both the
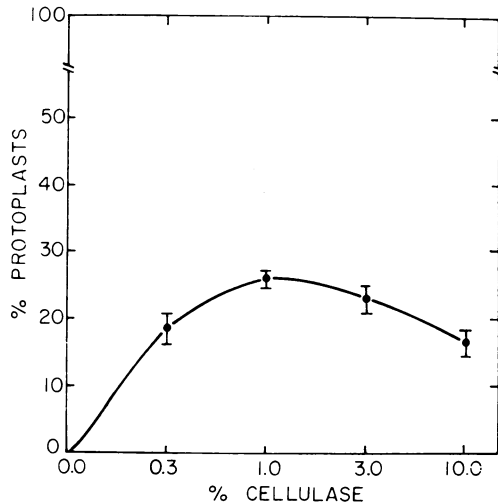
FIG. 4. Effect of cellulase Onozuka concentration on efficiency of protoplast isolation from tobacco cells. (Macerozyme supplied in 0.2% and mannitol at 0.7 M).
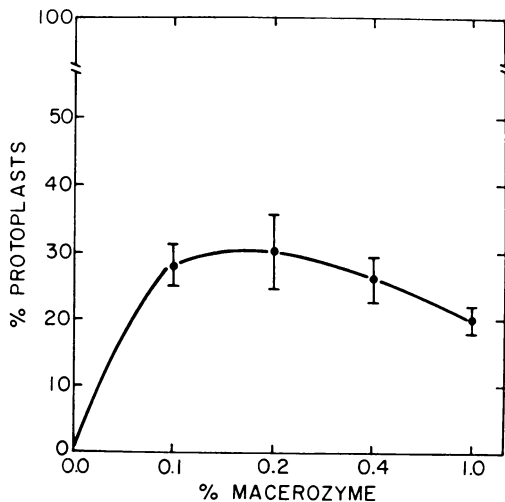

FIG. 5. Influence of Macerozyme concentration on efficiency of protoplast isolation from tobacco cells. (Cellulase Onozuka provided in 1% and mannitol at 0.7 M).

cells and the protoplasts among older samples. Note that in spite of diversity in cell shape, protoplasts were all spherical.

On the basis of the above information, cell cultures 4 to 5 days in a given passage were selected as best suited for protoplast isolation.

Concentration of Enzymes. The cellulase Onozuka concentration was tested in a range 0 to 10%, with Macerozyme being held constant at 0.2% and mannitol at 0.7 M. Similarly, the Macerozyme concentration was tested in a range 0 to 1.0%, with cellulase at 1%. Figure 4 shows that a 1.0% concentration of cellulase Onozuka was optimum. A lower concentration, 0.3%, was inadequate and higher concentrations, 3 and 10%, were without further benefit. This experiment showed that it was possible to reduce the amount of cellulase in the protoplast isolation solution to one-third of the originally prescribed concentration.

The optimum concentration of Macerozyme was spread over a wide range (Fig. 5). Equal yields of protoplasts, about 30% of the cells, were obtained with Macerozyme concentrations of 0.1, 0.2, and 0.4%. The 1% concentration appeared excessive.

A 0.2% Macerozyme concentration was selected as satisfactory.

pH of Enzyme Solution. The effectiveness of cellulase Onozuka and Macerozyme in producing protoplasts from tobacco cells was pH-dependent, as would be expected of enzyme-regulated processes. The optimum pH ranged between 4.7 and 5.7. A pH below or above this range clearly showed progressively and substantially lower yields of protoplasts. Of particular interest was the appearance of two maxima in the pH-dependent effectiveness of the enzymes. One occurred at pH 4.7 and the other at pH 5.7. The results can be seen in Figure 6. For all practical purposes, an initial pH in the range 4.7 to 5.7 was satisfactory; therefore, the pH of 5.7 was retained as standard.

Length of Incubation Period. The minimum incubation period appeared to be about 2 hr (Fig. 7). Moreover, incubation periods longer than 2 hr were without benefit; the yield of protoplasts remained in the neighborhood of 30% for periods ranging from 2 to 7 hr. Accordingly, a 2- to 3-hr incubation was selected as satisfactory for the isolation of protoplasts from tobacco cells.
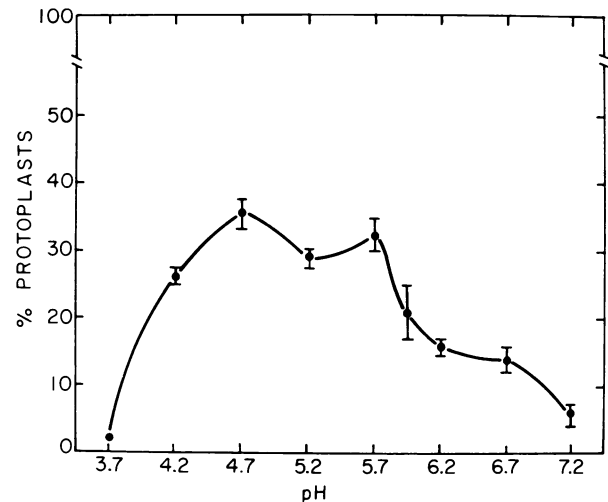

FIG. 6. Effect of pH of the enzyme solution on isolation of protoplasts from tobacco cells. The incubation mixture contained 1% cellulase Onozuka, 0.2% Macerozyme, and 0.7 M mannitol.
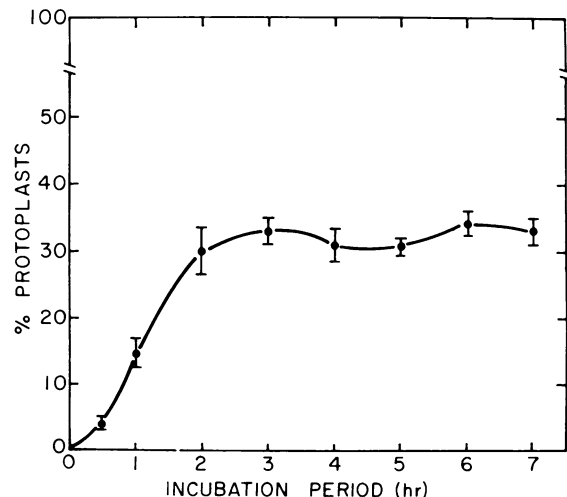

FIG. 7. Protoplast isolation as related to length of incubation period in enzyme solution. The incubation mixture was the same as in Fig. 6; pH 5.7; temperature 27 C.
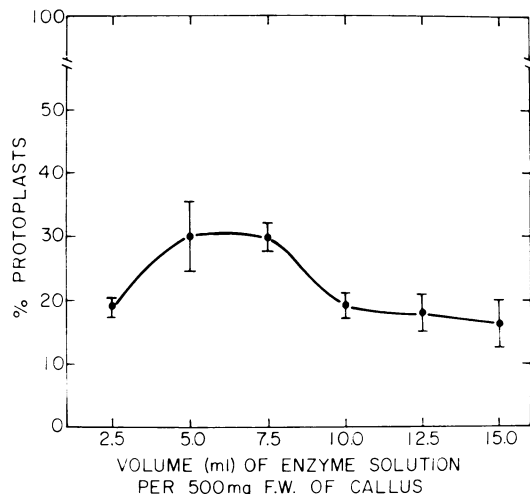
FIG. 8. Isolation of tobacco protoplasts as influenced by volume of enzyme solution. The incubation mixture was the same as in Fig. 7.
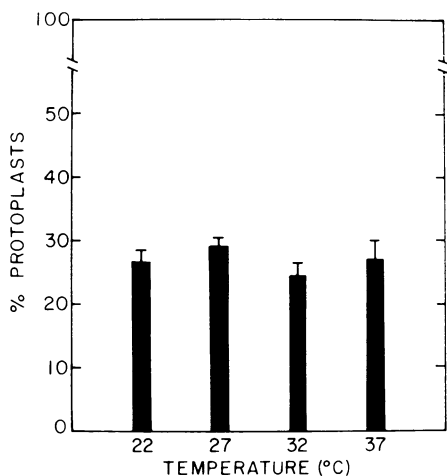
tion rate of 50 rpm, using the New Brunswick Model G-10 gyrotory shaker, was selected as standard.

Osmotic Requirement. An initial study was made to determine the optimum concentration of mannitol as the osmoticum in the incubation medium. The data from that study can be seen in Figure 11. The satisfactory concentration of mannitol extended over a wide range, from 0.3 to 0.8 M. Mannitol concentrations of 0.2 M and lower were clearly inadequate; the highest concentration presently examined, 0.9 M, was also less than satisfactory. The 0.7 M concentration of mannitol was retained as standard for the isolation of tobacco protoplasts.

Other soluble carbohydrates were examined next as substitutes for mannitol. They included glucose, fructose, galactose, sorbitol, and sucrose. Each was tested in 0.3 and 0.7 M concentrations. The results can be seen in Figure 12. With the exception of sucrose, which gave markedly poorer yields of protoplasts in either concentration, all carbohydrates tested gave quite favorable results in comparison to mannitol. The yields of protoplasts were in the neighborhood of 20 to 30% with these carbohydrates, as with mannitol. Also, with each of these substances, the higher concentration, 0.7 M, tended to

FIG. 9. Effect of incubation temperature on isolation of tobacco protoplasts. The incubation mixture was the same as in Fig. 7.
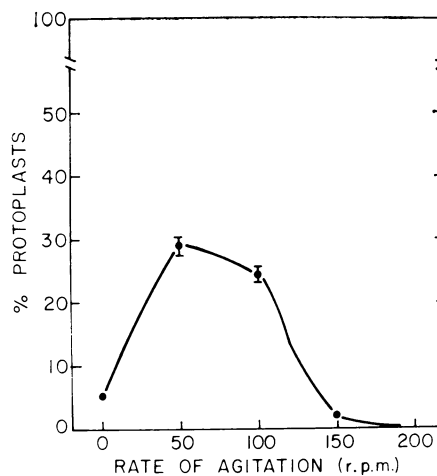
FIG. 10. Isolation of protoplasts from tobacco cells as determined by rate of agitation of the cell enzyme mixture. The incubation mixture was the same as in Fig. 7; 5 ml of enzyme solution and 500 mg cells contained in 50-ml Delong flask.

Volume of Enzyme Solution. Figure 8 shows the relationship between yield of protoplasts and volume of enzyme solution used. These volumes were considered in relation to 500-mg samples of tobacco cells. The maximum yields were obtained with enzyme solution volumes of 5.0 and 7.5 ml. A volume of 2.5 ml was perhaps insufficient, whereas those 10 ml and higher were excessive. The yields of protoplasts in either case was reduced from the maximum of 30% to 20%. As standard condition for the isolation of tobacco protoplasts an enzyme solution volume of 5 ml was selected.

Effect of Temperature. Surprisingly, there was no significant influence of temperature in the range 22 to 37 C (Fig. 9). The original 27 C was thus retained as standard.

Rate of Agitation. The incubation mixture containing enzyme and tobacco cells was agitated at various speeds, from 0 to 200 rpm, on a gyrotory shaker. As is evident in Figure 10, the highest percentage of protoplasts was obtained when the incubation mixture was agitated at 50 rpm. A 100 rpm agitation also gave a relatively high yield of protoplasts, but slightly less than the 50 rpm. Allowing the reaction mixture to remain stationary gave a very low yield of protoplasts. Similarly, agitation rates of 150 and 200 rpm were unsatisfactory and gave poorest proportions of protoplasts. The agita-

FIG. 11. Mannitol concentration and efficiency of protoplast isolation. The incubation mixture contained 1% cellulase Onozuka and 0.2% Macerozyme.

FIG. 12. Comparison of diverse carbohydrates as osmoticum in the isolation of tobacco protoplasts. The incubation mixture was the same as in Fig. 11; pH was 5.7.

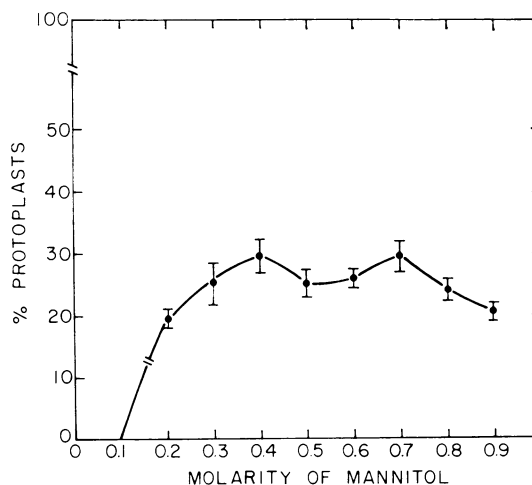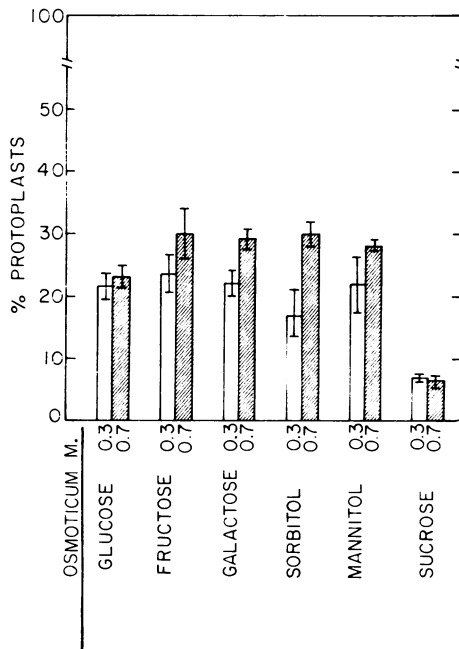produce a higher percentage of protoplasts. Since none of the carbohydrates showed improvement over mannitol as osmoticum, the latter was retained as the standard addendum of the protoplast isolation solution.

Other Addenda. Some investigators have included nutritive substances in their cell enzyme incubation medium (1, 3, 4, 7, 17). In this research no advantage was obtained by including Murashige and Skoog nutrient salts. Takebe et al. (18) included potassium dextran sulfate in their protoplast isolation medium. The results of an experiment with varying concentrations of potassium dextran sulfate incorporated into the medium for the isolation of protoplasts from tobacco cells can be seen in Table II. No significant improvement resulted from any addition of potassium dextran sulfate. Perhaps the highest concentration presently tested, 3%, caused some reduction in the yield of protoplasts.

Viability of Protoplasts. The isolation of protoplasts has little significance unless the procedure produces viable entities. The steps and conditions presently developed resulted in about 30% of the tobacco cells obtained from liquid suspension culture giving rise to protoplasts. Of these protoplasts approximately 80% regenerated cell walls in 4 days and some 40% resumed cell division and produced cell clusters within 8 days. Figure 13, a to d, shows the sequence of steps observed in the reconstitution of tissue from the protoplasts. The first step appeared to involve a volume increase of the protoplasts. This was followed by formation of the cell wall. Finally, the regenerated cell, although appearing spherical in contrast to the more angular characteristics of plant cells, divided repeatedly to give rise to a cluster.

DISCUSSION

The successful isolation of protoplasts has been described previously with several plants and with various kinds of tissues. The aim of this research was to explore systemically the significance of the conditions which have been frequently prescribed in procedures employed by various investigators. Although tobacco cells in liquid suspension culture have been

used as the experimental source of protoplasts, it is hoped that the information obtained will serve as a helpful guide in the development of procedures specially applicable to other plants and other types of tissues. The factors examined in this investigation have been as follows: (a) growth phase of the cells, (b) kinds and concentrations of cell wall degrading enzymes, (c) pH of the enzyme solution, (d) length of incubation period, (e) relationship between enzyme solution volume and quantity of cells utilized, (f) temperature effects, (g) rate of agitation of the enzyme tissue mixture, (h) kind and concentration of osmoticum, (i) influence of inorganic nutrients, and (j) requirement of potassium dextran sulfate. For purposes of comparison, the data obtained in this investigation, together with those which have been reported as satisfactory in some other researches, are summarized in Tables III and IV.

According to this investigation, tissues in the early growth phase with respect to the increase in cell number are probably most suitable as a source of cells for protoplast isolation. Throughout the late period of fresh weight increase, as well as during the later phase of increasing cell number, the yield of protoplasts was lower than during the early period of cell division. Eriksson and Jonasson (3) suggested that the cells in a frequently transferred suspension culture might produce protoplasts in higher frequencies. Schenk and Hildebrandt (17) prescribed tissues in the logarithmic phase of growth to be most desirable, although they did not specify whether the kinetic information was related specifically to cell division. In our cultures, the early logarithmic phase of increase in cell number was observed to be characterized by a preponderance of small and presumably thin-walled cells which probably contributed to the higher yield of protoplasts. During subsequent periods, the culture appeared to be composed of larger and more highly vacuolated cells, and perhaps cells with thicker walls. In extending the observation to other sources of plant cells, it may be suggested that, particularly with tissues taken directly from the plant, the age of the organ may play an important role in the isolation of protoplasts. Younger materials are probably better than older plant parts.

The source and kind of enzymes used in the isolation of protoplasts have been recognized in previous investigations as being decisive. It is also important to determine the optimum concentration of the enzymes. Most previous investigators have included cellulase Onozuka in the protoplast isolation mixture, and in a concentration ranging from 0.5 (4) to 5% (3, 10). In this investigation with tobacco cell suspension cultures 1% cellulase Onozuka has been found to be optimum and 5% has beeen clearly excessive. The 1% concentration of the Onozuka preparation corresponds to 3×10^5 cellulase activity units per ml of enzyme solution. This activity of the enzyme preparation was determined according to the method of Lewis and Varner (8). The procedures of other investigators (Table III) have included consistently the use of the enzyme preparation cellulase Onozuka, but not invariably of

Table II. *Effect of Potassium Dextran Sulfate on Protoplast Isolation*

Potassium Dextran Sulfate[1]	Protoplast
%	
0	25.4 ± 4.0
0.03	28.9 ± 1.4
0.3	28.6 ± 1.8
3.0	19.1 ± 1.8

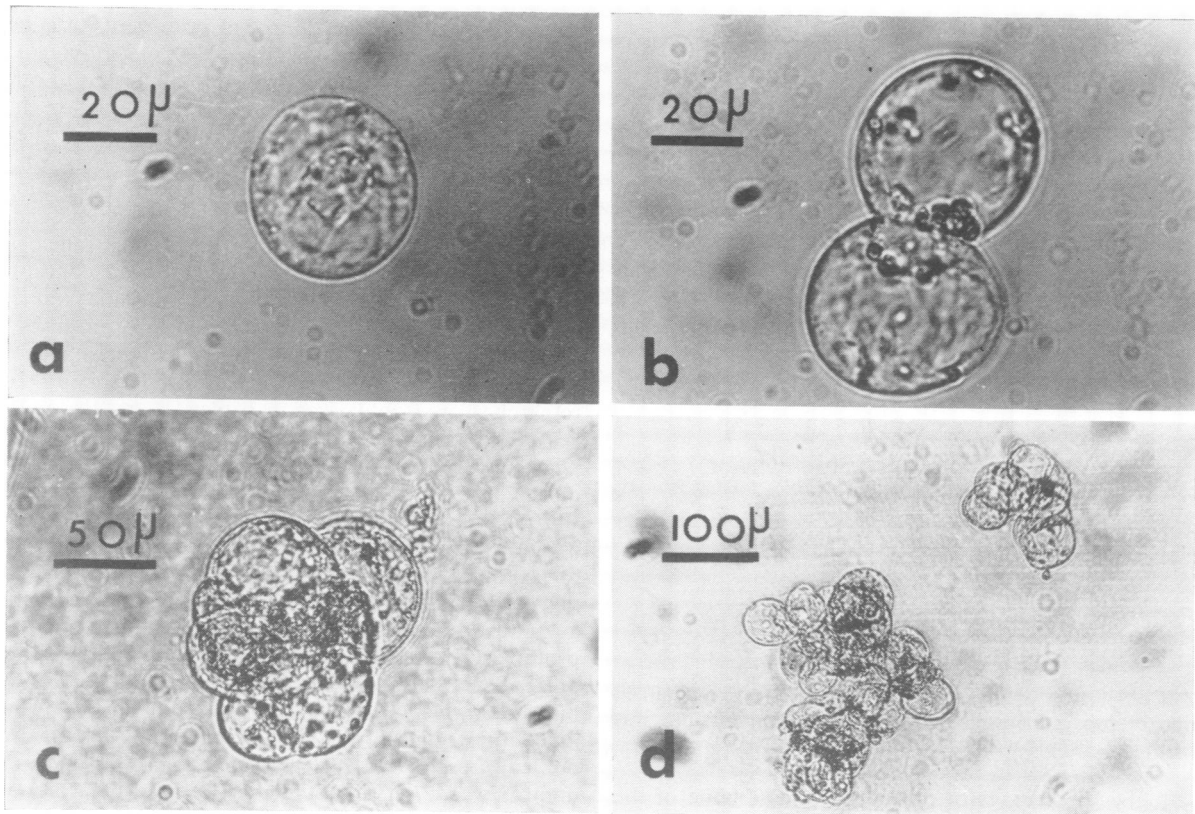[1] Meito Sangyo Co., Nagoya, Japan (lot No. RR-831-SK-3).

FIG. 13. Evidence of viability of tobacco protoplasts. a: A single protoplast; b: dividing cell after 5 days, c: cell cluster from regenerative cell after 10 days. d: cell cluster after 14 days.

Table III. *Comparison of Conditions Specified in Different Methods of Isolating Viable Plant Protoplasts*

Plant, Tissue, and Reference	Growth Phase of Tissue	Enzyme(s)	pH	Incubation Period
				hr
Nicotiana tabacum; callus; this paper	Early log phase of cell division	0.2% Macerozyme; 1% cellulase Onozuka	4.7 or 5.7	2–3
Arachis hypogea; callus; 17	Log phase	Purified cellulase	5.2	1½–2
Avena sativa; coleoptile; 15		Cellulase		1–2
Convolvulus arvensis; callus; 1		5% cellulase Onozuka		Overnight
Daucus carota; root; 6		0.1% pectinase, Sigma; 5% cellulase Onozuka		15
Daucus carota; callus; 4		0.25% hemicellulase Sigma; 0.5% cellulase Onozuka	5.5	10–12
Haplopappus gracilis; callus; 3		5% cellulase Onozuka	5.5–6.0	3–5
Glycine max; callus; 7		Pectinase Sigma; cellulase Onozuka	5.5	4–6
Nicotiana tabacum; leaf mesophyll; 18	Fully expanded leaves;	0.4% Macerozyme; 4% cellulase Onozuka	5.5	4
Nicotiana tabacum; leaf mesophyll[1]; 11	Fully expanded leaves;[1]	0.5% Macerozyme	5.8	
		2.0% cellulase Onozuka	5.2	2
Zea mays; callus; 10		2% Macerozyme; 5% cellulase Onozuka	5.4	3.5

[1] The procedure involves two steps: (*a*) maceration of tissue and (*b*) cellulase treatment.

Macerozyme. This study with tobacco cells has revealed that the latter enzyme preparation is as critical; cellulase Onozuka alone is completely ineffective in releasing tobacco protoplasts. Power and Cocking (13) also used the two enzymes for tobacco mesophyll protoplasts and Motoyoshi (10) used the combination of Onozuka cellulase and Macerozyme to obtain *Zea mays* protoplasts. Grambow *et al.* (4) apparently found a combination of cellulase Onozuka and either hemi-

cellulase Sigma or pectinase Sigma to be satisfactory for the release of protoplasts from *Daucus carota* cell cultures. Schenk and Hildebrandt (17) suggested that some commercially available cellulase preparations are contaminated with hemicellulase and/or pectinase and that these contaminants may be the reason for some cellulase preparation being effective when used alone. In the work reported by Takebe *et al.* (18) and Nagata and Takebe (11), the enzyme preparations cellulase

Table IV. *Comparison of Conditions Specified in Different Methods of Isolating Viable Plant Protoplasts*

Plant Tissue, and Reference	Enzyme Solution Volume	Incubation Temperature	Agitation	Osmoticum
		C		
Nicotiana tabacum; callus; this paper	5 ml/500 mg tissue	22–37	50 rpm	0.3–0.8 M mannitol
Arachis hypogea; callus; 17			Gentle agitation	KNO_3; $CaCl_2$
Avena sativa; coleoptile; 15		25	Gentle agitation	0.5 M mannitol
Convolvulus arvensis; callus; 1		29		0.4 M sorbitol
Daucus carota; root; 6	10 ml/g tissue	30		3.5% KCl; 0.5% $CaCl_2$
Daucus carota; callus; 4	4 ml/100 mg tissue	Ambient		0.56 M mannitol or sorbitol
Haplopappus gracilis; callus; 3		30	Gentle swirling	0.4–0.6 M sorbitol
Glycine max; callus; 7				0.1 M sucrose; 0.3 M sorbitol
Nicotiana tabacum; leaf mesophyll; 18	20 ml/2 g tissue	21	None	20% sucrose
Nicotiana tabacum; leaf mesophyll[1]; 11	25 ml/2 g tissue	25	120 rpm	0.7 M mannitol
		36	Gentle swirling	0.7 M mannitol
Zea mays; callus; 10	28 ml/500 mg tissue	37	60 rpm	0.6 M mannitol

[1] The procedure involves two steps: (a) maceration of tissues and (b) cellulase treatment.

Onozuka and Macerozyme were used to isolate protoplasts from tobacco mesophyll. However, these enzyme preparations were not employed in a common mixture; they were used in separate but sequential steps—first Macerozyme, then cellulase Onozuka. In the present study with *Nicotiana tabacum* cells, a 0.2% concentration of Macerozyme was about optimum when used in conjunction with 1% cellulase Onozuka.

The study of enzyme solution pH showed that there are two optima with respect to pH: one occurs at about 4.7 and the other in the neighborhood of 5.7. This phenomenon has been observed consistently in several repetitions of the experiment, indicating that the result was not due to chance. These optima probably indicate the presence of two isozymes of cellulase, as recently discovered with *Tricoderma viridi* cellulase by Linkins and Lewis (9), or different pH requirements of the preparations cellulase Onozuka and Macerozyme. According to Linkins and Lewis (9), the *Tricoderma* cellulase is composed of two isozymes, differing in protein molecular weights and isoelectric points. Sheldrake (16) has shown that the cellulase preparation obtained from the abscission zone of *Acer pseudoplatanus* is characterized by two pH optima with respect to rates of enzyme activity; one is apparent at pH 5.3 and the other at 5.9. The available information, therefore, does indicate possible involvement of isozymes with different pH optima.

The period of incubation of cells with enzyme solution has varied considerably among investigators. Reusink and Thimann (15) used a period of 1 to 2 hr for *Avena sativa* coleoptile, whereas Grambow *et al.* (4) routinely used an incubation time of 10 to 12 hr for *Daucus carota* callus. Our work with tobacco disclosed that a period of 2 hr is sufficient and longer incubation periods are without additional benefit. The fact that protoplast isolation in this study involved relatively small and recently divided, and therefore thin walled cells, may be the reason for the shortness of the required incubation period. Variations due to plant genera and other factors not excluded, however.

While temperature was expected to influence significantly the yield of protoplasts, tests have shown that at least in the present case no significant difference in efficiency was observed among incubation temperatures ranging from 22 to 37 C. This is surprising since enzyme activity is often dependent on temperature. In contrast, enzyme solution volume in relation to tissue volume, not anticipated to play a major role, has been a

Table V. *Efficiency of Different Methods of Isolating Protoplasts from Tobacco Callus*

Isolation Method	Protoplasts
	%
Eriksson and Jonasson (3)	10.5 ± 1.2
Grambow *et al.* (4)	22.3 ± 3.9
Kameya and Uchimiya (6)	6.1 ± 0.7
Motoyoshi (10)	13.3 ± 3.9
Power and Cocking (13)	6.8 ± 0.6
Takebe *et al.* (18)	9.0 ± 0.6
This paper	29.0 ± 0.7

critical factor. In this research, using a 500 mg quantity of tobacco cells, the highest yield of protoplasts was obtained when the enzyme solution was provided in volumes of 5.0 and 7.5 ml. Lower and higher quantities of solution gave substantially fewer protoplasts. This finding is within the range of some other investigators, but differs significantly from conditions described by many others (Table IV). Motoyoshi (10) used 28 ml of enzyme solution per 500 mg cells and Grambow *et al.* (4) used an equivalent of 20 ml. The use of larger volumes of solution by other investigators may simply reflect the involvement of still other factors, including different shape and size of the reaction vessel, and different rates of agitation.

The rate of agitation of the cell enzyme mixture is evidently very important in determining the degree of success in isolating plant protoplasts. When using tobacco cells obtained from liquid suspension cultures, the optimum agitation rate under our conditions was 50 rpm. Substantially lower or higher rates of agitation were unsatisfactory. At very high rates, *e.g.*, 150 rpm, many of the protoplasts were damaged. It is interesting to note that Motoyoshi (10) used 60 rpm to agitate the reaction mixture in isolating *Zea mays* protoplasts. Takebe *et al.* (18) and Nagata and Takebe (11) prescribed 120 rpm for the initial Macerozyme treatment step and gentle swirling for the cellulase step.

Perhaps as one might expect, the osmoticum concentration in the cell-enzyme mixture has been consistent among different investigators (Table IV). The usual range previously has been from 0.4 to 0.7 M. The study with tobacco cells disclosed that the range may be extended; virtually equal success in protoplast isolation has been obtained with the osmoticum provided

in concentrations varying from 0.3 to 0.8 M. Either sorbitol or mannitol was used as the osmoticum by other investigators. Our study showed that several soluble carbohydrates, except sucrose, were equally satisfactory. They include glucose, fructose, galactose, sorbitol, and mannitol. Schenk and Hildebrandt (17) and Kameya and Uchimiya (6) were successful in satisfying the osmotic needs with salts, such as KNO_3, KCl, and $CaCl_2$. This research with tobacco cells disclosed that potassium dextran sulfate as well as nutrient salts was unnecessary as an addendum to the enzyme solution involved in the isolation of protoplasts.

A successful procedure of isolating plant protoplasts must result in a substantial yield of protoplasts. More important, it must produce protoplasts which are capable of reforming into cells under suitable conditions. The present procedure enabled the release of protoplasts from about 30% of tobacco cells and cell aggregates obtained from liquid suspension cultures. For comparative purposes the efficiencies in yield of protoplasts from tobacco cells in suspension culture by methods of other investigators are shown in Table V. The efficiency of the presently developed method for tobacco cells was at least 1.5 times better than some procedures and as much as 5 times superior to others. When returned to a suitable culture medium, 80% of the protoplasts regenerated cell walls and 40% resumed cell division activity, indicating that a significant proportion of the protoplasts obtained by our method was viable.

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