CLONAL VARIATION IN PARAMECIUM. I. PERSISTENT UNSTABLE CLONES¹

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ABSTRACT

Clones of *Paramecium* of identical serotype when cultured in test tubes may differ in their ability to give rise to subclones of this serotype. Characteristically, stable clones yield progeny indistinguishable from their parents, while from unstable clones diverse subclones with new serotypes can be isolated repeatedly. Stable lines are resistant to changes in culture medium and also are unaffected by most sera. In contrast, the numbers and kinds of serotypes displayed among subclones derived from unstable lines are often affected by these same agents. Stable and unstable clones are interconvertible when the medium from individual cultures is repeatedly and frequently replaced by fresh culture fluid. This effect is very likely a result of the removal of the initial exhausted medium with any cell products rather than the addition of fresh nutrient.

OF the several intriguing aspects of the *paramecium* serotype system, the one that is perhaps the most significant is the prolonged, continuous existence of different antigens in cells of the same genotype (FINGER *et al.* 1972).

Although generally there is one particular set of conditions that favors one serotype rather than another (growth rate, temperature, etc.), it is not difficult to maintain different serotypes under apparently identical conditions (BEALE 1954). Since such cells regularly and repeatedly divide in this situation, this phenomenon may be couched in more general terms as an example of the transfer of a specific, differentiated state through many generations. Another example of the extended maintenance of two different phenotypes in cells of the same genotype under the same environmental conditions is presented below.

All of the cells comprising a clone may maintain the same serotype when cultured together in tubes. However, these same cells when isolated from each other in depression slides can yield subcultures which contain cells of a new serotype. Thus, by one criterion a clone may be considered stable (when grown in tubes), by another unstable (when grown in a slide). When the original cells are allowed to multiply and grow in culture tubes, the initial serotype persists in both kinds of clones. There are, of course, a number of differences between

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the environments of a single cell (or a few cells) in a depression with one ml of culture fluid and the same cell(s) in a 20 ml test tube culture along with hundreds of other cells, often congregating near the surface when well fed. We have reported experiments indicating that the serotype assumed by an unstable cell may be due, in part, to substances released by other cells (FINGER 1968). From these and other observations a hypothesis invoking inhibitors of specific antigen synthesis has been proposed (FINGER 1967). One prediction arising from the hypothesis is that stable clones should become unstable, and vice versa, simply by continuous removal of cell products.

The experiments described here demonstrate that the two kinds of clones differ in their responsiveness to changes in their environment, such as the introduction of serum and the replacement of their culture medium. Also, clones can change their stability when grown at maximum fission rate, a condition achieved by repeated replacement of medium by fresh culture fluid. Evidence is also presented that this change is due to removal of cell products and not to the addition of nutrients.

MATERIALS AND METHODS

Stocks and culture: Stock 7 of syngen 2, *Paramecium aurelia*, was used exclusively. All clones and subclones were vegetative descendants of a single cell isolated in Pinehurst, North Carolina (FINGER 1957). Cultures were maintained in a Cerophyl medium (FINGER and HELLER 1962) at 17°C. Unless otherwise stated, test tube and flask cultures were replenished with fresh medium weekly. Exogenous bacteria were not added to the culture medium. All serotypes arose spontaneously within stock tubes maintained at 17° or 27°. For maintaining clones under conditions allowing maximum fission rates, about 1.5 ml of well-fed cells from the topmost portion of a 150 × 16 mm culture tube was removed, the remaining medium discarded, and the cells returned to the original tube, which was topped up with fresh medium. For cultures maintained at 27° this procedure was followed daily; at 17°, every other day. In those experiments in which cells were transferred to an isotonic salt solution lacking growth factors, DRYL's (1959) solution was used: sodium citrate 0.1 M, 10 ml; sodium phosphate dibasic 0.1 M, 10 ml; calcium chloride 0.1 M, 15 ml; and distilled water 945 ml.

Classification of clones for serotype and stability: Appropriately diluted antisera (1:100 or 1:200 for homologous serotypes) were added to samples of cultures containing 20–100 cells. After 1–2 hr the cells were examined microscopically for immobilized paramecia. A culture was classified as "C" if any cells were immobilized by a C antiserum, "G" if any were immobilized by a G antiserum, etc. Three kinds of antisera were employed (G-, C-, and X-immobilizing). A culture most or all of whose cells were unaffected by any of these antisera was classified as "Z" (FINGER *et al.* 1969).

To determine whether a clone was unstable, two aliquots (ten drops each) were removed from a test tube culture. Ten drops of medium from the original culture were added to one sample and 10 drops of fresh culture fluid to the other. The following day 60 isolates from each mixture were made into fresh culture fluid in depression slides (Bel-Art Products, Pequannock, New Jersey). About 0.5 ml of fresh medium was added every two or three days until a population of 200–500 paramecia per subclone was reached (usually about 5–7 days after the original isolate had been made), at which time the contents of a depression were divided into three aliquots for testing with the three antisera. In the case of stable clones both aliquots yielded subclones nearly exclusively of the original serotype. In contrast, subclones from unstable clones generally were of at least two types: those pure for the original serotype and those that were mixtures of the original serotype and one or more new serotypes.

When a difference existed between the two aliquots in the proportions of subclones with new

serotypes, this difference provided a reliable guide for what could be expected on continued sampling of the original culture tube (FINGER 1968). Often one serotype appeared with greater frequency among the subclones from the aliquot to which had been added fresh culture fluid. Almost invariably this serotype, when a second sampling was taken a day later from the original culture tube, would occur with even greater frequency. Thus, when a serotype appeared more often among the subclones from the aliquot with fresh culture fluid (symbolized by the notation $\Delta = +$), this was taken as an indication that this serotype would be made by more subclones from a similar sample taken the following day. Conversely, when the number of subclones containing a particular serotype from the exhausted medium aliquot was larger than that from the aliquot with fresh medium ($\Delta = -$), the 48-hr sample from the original tube would yield fewer subclones containing this serotype than the 24-hr sample.

Immediately before individual paramecia were isolated, the original clone was tested for serotype. Only clones 90–100% pure for a serotype were used in these experiments. Test tube cultures were maintained at 17°C, depression slide cultures at room temperature in moist chambers.

Antisera: The diagnostic sera used were obtained from rabbits after immunization with purified immobilization antigen (FINGER and HELLER 1962). Other antisera, devoid of immobilizing antibodies, that were used in some experiments were preimmune sera or antisera from animals injected with cell-free concentrated culture fluid in which paramecia had been grown (FINGER *et al.* 1969), or a cell fraction which was also present in such a conditioned medium.

About 2.5 ml of either antigenic material were combined with 2.5 ml of complete Freund's adjuvant and injected into the footpads of a rabbit. After five weeks a 0.2 ml booster injection was given intravenously, and bleedings were initiated four days later.

RESULTS

For many months, both stable and unstable clones can maintain their ability to give rise, respectively, to pure subclones and mixed subclones. Repeated tests of the original test tube cultures during this time revealed no changes in the serotypes of the parental cultures.

One example of a stable clone of the C serotype is clone 18 (Table 1). Throughout almost a full year this clone yielded only 25 subclones (of a total of 449) which were not exclusively C. Similar observations also were noted for clones of serotypes G and X.

Under the same growth conditions unstable cultures retained their ability to yield mixed subclones. For example, although by immobilization tests clone 32 was indistinguishable from clone 18, it repeatedly gave rise to subclones with G and X cells, as well as C cells (Table 1). Although generally unstable clones did not persist as long as did stable clones, many could maintain their character for 8–12 months at 17° (more than 100 fissions). Not only did the stability character persist, but the particular pattern of subclones that arose among the isolates from individual experiments often persisted also. Thus, clone 23 (initially X) usually produced largely X subclones with a minority class of G subclones; clone 32 invariably yielded chiefly G- and C- containing subclones; and initially G clone 40, although more variable than the others, nevertheless generally tended to produce G as well as X subclones.

Another kind of unstable clone called "Z" is composed of cells which do not react with any of the G, C, or X antisera. As has previously been reported (FINGER *et al.* 1969), these clones are usually unstable and among subclones

TABLE 1
Persistence of clonal stability trait

Date of subclone isolation	Number subclones tested	Number of subclones with serotypes			
		G	C	X	Z

UNSTABLE CLONES					
Clone 32—C serotype in test tube culture					
April 9, 1969	40	24	22	2	0
April 21, 1969	68	28	22	4	24
April 24, 1969	69	33	42	2	0
March 5, 1969	81	25	60	3	7
January 22, 1970	52	27	42	8	2
February 4, 1970	55	15	43	7	1
February 12, 1970	57	24	40	14	0
February 19, 1970	57	21	45	7	1
February 26, 1970	58	40	39	20	2
Clone 40—G serotype in test tube culture					
July 4, 1969	58	54	1	30	6
January 22, 1970	55	3	20	6	55
February 4, 1970	49	29	0	48	3
February 12, 1970	53	44	11	28	1
February 19, 1970	58	54	1	30	6
February 26, 1970	57	56	2	9	7
Clone 23—X serotype in test tube culture					
April 9, 1969	66	21	9	63	3
April 21, 1969	75	9	2	67	5
April 24, 1969	66	11	1	57	2
April 7, 1970	59	4	0	50	12
Clone 20—Z serotype in test tube culture					
April 21, 1969	78	19	5	14	49
April 24, 1969	82	30	18	10	38
July 4, 1969	56	1	32	1	22
September 23, 1969	41	39	7	3	2
September 29, 1969	48	28	24	6	1
Clone 12—Z serotype in test tube culture					
July 4, 1969	51	14	1	18	35
January 22, 1970	59	14	0	16	44
February 4, 1970	51	2	3	4	47
February 12, 1970	54	2	0	9	48
February 19, 1970	57	14	1	18	35
February 26, 1970	50	18	0	18	32

STABLE CLONES					
Clone 18—C serotype in test tube culture					
April 9, 1969	49	3	44	5	3
April 24, 1969	79	0	78	0	1
July 4, 1969	58	0	58	0	3
January 22, 1970	51	0	51	1	0
February 4, 1970	53	1	50	3	1
February 12, 1970	50	0	49	0	1
February 19, 1970	58	0	58	0	3

TABLE 1—Continued

Date of subclone isolation	Number subclones tested	Number of subclones with serotypes			
		G	C	X	Z
Clone 42—G serotype in test tube culture					
September 23, 1969	55	52	0	0	0
September 29, 1969	52	52	0	0	0
October 6, 1969	41	41	1	0	0
January 22, 1969	51	50	0	3	1
June 1, 1970	58	57	2	0	0
Clone 123—X serotype in test tube culture					
July 13, 1966	45	0	0	45	0
December 18, 1967	49	1	4	46	1
January 2, 1968	46	3	2	44	2
September 9, 1968	50	0	0	50	1

* In these and other tables, the number of subclones with serotypes G, C, X, and Z usually totals more than the number of subclones tested because when a clone contains two or more kinds of serotypes it will be classified under several categories.

isolated from test tube cultures are found G, X, and C cells. Z clones, in contrast to G, C, and X unstable clones, yielded patterns of serotypes among derived subclones that varied considerably from experiment to experiment.

Effect of overnight suspension in fresh medium: In order to classify a clone as stable or unstable, individual paramecia must be placed into fresh culture fluid so that a population may be generated. Nevertheless, even when a paramecium is allowed to remain in a test tube with other members of its clone, an effect of fresh medium may be demonstrated. This is done by simply replacing the "used" medium with new culture fluid about 24 hr before isolates are made and comparing the kinds of subclones produced with those produced from a tube whose "used" medium was not replaced. To determine whether an individual clone was affected by transfer to fresh medium, four aliquots of test tube cultures were used in each experiment. Only data from those experiments in which duplicate tubes yielded proportions of subclones in agreement with each other (judged by a 2×2 chi-square test for homogeneity at the $P = .05$ level) were analyzed. If replicate experiments were determined as yielding results expected on the basis of repeated sampling from a single population, the data from duplicates were combined and the new totals used in any comparison. Fewer than one in five sets effect (Table 2). In comparison, subclones from unstable clones (half of more than two dozen studied) often express different serotypes depending on whether of duplicates exhibited a statistically significant heterogeneity.

With stable clones overnight exposure to new medium has no demonstrable or not they had been maintained previously in fresh culture fluid (e.g., clones 44, 1, 40, 98, and 25 (Table 2)). This effect is most likely due to the removal of something present in exhausted medium. The fresh medium probably acts as a diluent since its action can be duplicated with a salt solution (see below).

Response of stable and unstable clones to serum: Immobilizing antiserum, other kinds of antisera and preimmune serum can all induce transformation (FINGER

TABLE 2

Effect of overnight suspension in fresh culture fluid

Clone	Suspension medium	Serotype				
		Number	G	C	X	Z
A. UNSTABLE CLONES						
44(Z) *	Fresh	60	4	32	19	20
		58	3	30	20	19
	Old	58	5	27	31	11
		58	0	23	29	17
	P		>.05	>.10	<.01	>.10
15(Z) *	Fresh	60	1	1	11	57
		59	0	3	19	47
	Old	60	0	0	12	53
		59	0	0	19	53
	P		>.30	>.10†	>.80	>.50
1(Z) *	Fresh	58	4	1	14	50
		59	5	1	9	53
	Old	57	9	2	19	41
		60	7	4	21	42
	P		>.10	>.10	<.05	<.01
14(Z) *	Fresh	52	4	6	16	39
		48	4	1	18	34
	Old	47	8	0	18	36
		48	5	1	11	38
	P		.20	>.05†	>.50	>.50
25(Z) *	Fresh	57	21	52	2	0
		59	15	50	1	0
	Old	58	7	55	0	0
		58	12	58	0	0
	P		<.01	<.01	>.05	
21(G) *	Fresh	57	43	0	15	5
		56	40	0	14	8
	Old	59	45	0	10	7
		57	41	0	8	0
	P		>.70		>.05	>.10
23(X) *	Fresh	59	4	0	50	12
		57	3	0	47	8
	Old	57	8	0	44	12
		55	3	0	40	15
	P		>.20		>.10	>.20
47(X) *	Fresh	59	4	14	52	0
		58	2	6	57	0
	Old	53	6	5	53	0
		53	5	5	50	2
	P		.20	>.05	>.10	

TABLE 2—Continued

Clone	Suspension medium	Serotype				
		Number	G	C	X	Z
32(C)*	Fresh	49	21	42	5	4
		52	27	42	8	2
	Old	53	31	39	10	0
		50	27	42	18	0
	P		>.20	>.30	<.05	<.05†
98(C)*	Fresh	55	15	42	4	4
		60	18	56	2	1
	Old	51	0	48	1	3
		58	4	54	4	0
	P		<.001	<.05	>.05	>.05
40(G)*	Fresh	49	29	0	48	3
		56	30	0	53	4
	Old	53	31	0	46	7
		56	22	0	44	10
	P		>.20		<.01	>0.50†
21(G)*	Fresh	54	18	18	21	3
		47	18	22	11	2
	Old	59	27	29	26	2
		58	29	15	20	4
	P		>.05	>.30	>.20	
B. STABLE CLONES						
42(G)*	Fresh	58	57	2	0	0
		55	55	0	0	0
	Old	57	57	0	0	0
		54	54	1	0	0
48(G)*	Fresh	44	44	0	1	0
		43	43	2	2	0
	Old	51	50	0	0	1
		50	48	0	1	1
48(G)*	Fresh	60	59	2	1	1
		60	59	1	1	1
	Old	53	53	3	1	0
		56	56	1	3	0
42(G)*	Fresh	51	50	0	3	1
		54	54	0	2	1
	Old	54	54	0	1	0
		50	49	0	3	0
18(C)*	Fresh	58	2	56	2	0
		59	1	59	1	1
	Old	56	0	55	1	0
		60	0	59	0	1

TABLE 2—Continued

Clone	Suspension medium	Serotype				
		Number	G	C	X	Z
15(C)*	Fresh	57	0	55	2	0
		60	1	59	0	0
	Old	56	0	54	1	1
		60	0	58	3	0
36(C)*	Fresh	53	2	51	0	1
		51	1	50	0	0
	Old	54	1	54	2	0
		55	0	54	1	0
1(C)*	Fresh	58	1	58	0	0
		60	1	60	0	0
	Old	59	4	58	4	0
		57	6	56	4	0
28(X)*	Fresh	57	6	0	54	3
		56	0	0	52	5
	Old	57	3	1	55	1
		57	4	1	54	3

* Initial serotype.

† Yates' correction applied.

et al. 1969). To compare the effect of these sera on stable and unstable clones, cultures were exposed to dilute serum overnight, and subclones were initiated from those cultures whose original serotype was unaltered, i.e., untransformed cells. Both stable and unstable clones could be affected by serum, although generally stable clones were more resistant. For example, of three unstable clones all were affected by immobilizing antibodies, while only one of four unstable cultures responded to any serum (Table 3). Furthermore, when a serum did induce transformation, generally the effect was most pronounced with unstable clones.

In brief, stable clones are characterized by the persistence among subclones of the serotype possessed by the original clone, and a resistance to changes in the environment, such as the substitution of fresh culture fluid or serum for exhausted medium. In contrast, unstable clones are susceptible to these environmental changes despite the fact that, like stable clones, unstable clones retain their original serotypes for many generations when grown in mass cultures.

Induced changes in clonal stability: A simple way to replace "used" culture fluid depleted of bacteria and growth factors by dividing paramecia is to remove approximately one ml of a ring of cells from the top of a test tube and discard the remainder. The cells in this sample (usually several thousand from a well-fed culture) are then transferred to about 18 ml of fresh culture fluid. This procedure can be repeated daily, for cells grown at 27°, and every other day, for cells at 17°, for extended periods of time without fear of losing the culture, i.e., the cells are reproducing about as rapidly as they are being removed.

In the experiment summarized in Table 4, 60 cells were isolated from each of

TABLE 3

Effect of antiserum on stable and unstable clones

		Number of subclones tested	Number subclones with serotype			Z
			G	C	X	
UNSTABLE CLONES						
Clone 4—Initially C						
Culture fluid		60	7	31	27	3
		55	6	33	17	0
Antiserum	Antibodies against†					
1162	G, X	60	1*	55*	18	0
		59	1	49	19	0
1154	C, X	60	13	2*	39*	12*
		60	12	1	42	17
1210	Fraction of medium	57	0*	0*	56*	0
		58	0	1	54	5
Clone 101—Initially X						
Culture fluid		58	16	0	42	3
		59	11	0	53	2
1163	G, X	58	13	1	39	9*
		59	5	1	42	14
1154	C, X	60	37*	0	28	4
		60	47	0	28	1
1213	Fraction of medium X	57	11	0	39	9*
		59	23	0	20	19
Clone 36—Initially G						
Culture fluid		56	50	10	13	0
		57	48	16	15	0
Antiserum	Antibodies against†					
1162	G, X	58	50	13	28*	0
		60	51	11	34	0
1163	G, X	58	42*	15	23*	0
		56	30	14	26	1
1165	G	59	30	8	39*	1
		56	17	7	43	1
1202	Fraction of medium, G	56	36	10	36*	0
		57	24	6	42	1
STABLE CLONES						
Clone 45—Initially C						
Culture fluid		59	5	55	3	0
		60	1	57	1	0
1193	C	59	1	54	4	2
		59	0	56	4	2
1157	C	60	1	59	0	0
		60	1	58	2	1
1176	C, G	59	0	57	3	0
		59	0	58	1	0

TABLE 3—Continued

		Number of subclones tested	Number subclones with serotype			
			G	C	X	Z
Clone 97—Initially C						
Culture fluid		57	3	55	2	0
		60	4	59	2	0
Antiserum	Antibodies against†					
1162	G, X	59	1	56	9*	0
		60	1	58	7	0
1154	C, X	60	8*	50	17*	0
		59	10	53	9	0
1210	Fraction of medium	60	6	51	11*	0
		59	4	51	10	0
Clone 12—Initially X						
Culture fluid		60	3	0	56	2
		57	3	1	54	1
1212	Fraction of medium	58	1	0	57	0
		60	0	1	58	2
1213	Fraction of medium	59	2	0	56	8
		56	3	0	53	3
1214	Fraction of medium, X	60	0	0	58	3
		55	1	0	52	2
Clone 37—Initially G						
Culture fluid		57	57	0	0	0
		59	59	0	1	0
Antiserum	Antibodies against†					
1203	Fraction of medium	59	57	0	7*	0
		58	58	0	3	0
1165	G	59	59	0	0	0
		57	57	0	0	0

* Number of subclones of this serotype differ significantly from those observed after exposure to culture fluid (χ^2 analysis at a P level of .05, or less). Only data from those experiments in which duplicate tubes yielded proportions of subclones in agreement with each other (judged by a 2×2 chi-square test for homogeneity at the $P = 0.5$ level) were used.

† The presence of G, C, or X antibodies was determined by immobilization tests. Some sera were made against a fraction of a saline-alcohol extract of cells. Those having antibodies which precipitate with an extract of medium in which paramecia have lived are denoted by the phrase "fraction of medium." When these antisera also immobilize, it is so indicated.

18 cultures at day 1. After two weeks of continuous refeeding at 17° or 27°, another sample of 60 individuals was removed. The subclones derived from each isolate were tested for serotypes after about a week's growth in depression slides. The kinds of serotypes present in each subclone were noted as well as whether the subclone was pure for a particular serotype. Only cultures which at the outset of the experiment were 90–100% of one serotype and which at the conclusion of the continuous feeding segment of the experiment (when the second sampling

TABLE 4

Conversion of stable and unstable clones by rapid growth

	Percent subclones containing cells of initial serotype		Percent subclones composed entirely of initial serotype*	
	Beginning of experiment	After rapid growth	Beginning of experiment	After rapid growth
STABLE CLONES				
7-90	100	100	100	100
7-91	100	100	100	100
7-93	100	100	100	100
7-109	95	93	95	72
7-116	100	100	100	100
7-111	100	100	98	100
7-117	100	100	100	95
7-49	100	93	100	44
UNSTABLE CLONES				
7-101	100	82	35	71
7-106	66	73	7	78
7-110	57	94	79	36
7-5	98	97	80	61
7-7	96	93	62	79
7-1	93	100	74	97
7-3	87	96	72	96
7-52	87	74	61	73
7-31	84	87	24	68
7-29	74	82	39	55

was made) had not transformed from the original serotype were followed. Both parameters—the proportion of subclones with any cells of a particular serotype and the percentage of these subclones that were exclusively of this serotype—are recorded, since both serve as criteria for stability. The latter parameter is a particularly sensitive indicator of serotype change.

Among the cultures listed in Table 4, only 7-49 of the two stable clones (7-49 and 7-4) was induced to become unstable, as judged by the percentage of pure subclones (the last column) among those with cells of the original serotype. All of the unstable clones, by this same criterion, became more like the stable clones on repeated removal of exhausted medium. In other experiments (cf., Table 6), the same pattern emerged: stable clones became unstable rather infrequently; unstable clones generally grew more stable.

Among the unstable clones an interesting correlation was noted. Those cultures which yielded a decreased number of subclones of the initial serotype from successive daily samples during normal growth ($\Delta = -$; cf., MATERIALS AND METHODS) became more stable under the new conditions of continual transfer to fresh culture fluid (Table 5A). This phenomenon was not restricted to the original serotype. Any serotype which ordinarily would diminish in frequency among isolates under the usual feeding conditions (fresh culture fluid once a week at

TABLE 5

Effect of changing culture fluid on serotypes of subclones isolated from unstable clones

Δ of initial serotype*	A Number of clones in which initial serotype			Percent clones whose serotype increased with continual feeding
	Increased	Decreased	Did not change	
+	1	8	4	7.7
—	18	3	3	75.0
0†	3	13	4	15.0

Δ of serotype	B Number of clones in which any new serotype increased	Number of serotypes tested	Percent clones whose serotypes increased with continual feeding
+	5	37	13.5
—	38	46	82.6
0	40	135	29.6

* See MATERIALS AND METHODS for meaning of Δ .

†0 = No difference between subclones isolated from culture kept overnight in original medium and culture transferred to fresh medium.

17°), increased in frequency when the parental clone was transferred to fresh medium every other day (Table 5B).

Clearly, whether a culture is fed often or occasionally has a pronounced effect on the kinds of serotypes that the culture may give rise to, without any outward transformation in the original culture. Unfortunately, the means by which these changes are induced does not lead to a single explanation. Removing cells from their original medium obviously dilutes out the medium, but because cells in order to thrive must be placed in fresh culture medium it would seem that the two operations—dilution and the addition of fresh nutrients—cannot be divorced from each other.

To distinguish between these alternatives, two different kinds of experiments were carried out. In one experiment, cultures were divided into two sets at 27°. One set was “ringed” and fed at 9:00 a.m. and again at 3:00 p.m. every day for 12 days. The other set was fed daily at 3:00 p.m. only. In all cases, the cultures were dividing maximally, or nearly so, so that the chief difference between the two sets was that “old” medium was removed twice as often in the first set.

If the effect experienced by cells transferred to fresh culture fluid was due to the maximal growth rate achieved through added nutrient, there should be no difference between the two sets. On the other hand, if the consequence of changed medium was due to removal of old medium (presumably containing cell products) then the cultures fed twice a day should, if initially stable, become more unstable than those fed once a day. Similarly, those unstable clones fed twice daily should become more stable than the same clones fed only once daily.

That the effect of transfer of cells from old to new medium is the result of dilution is supported by the data in Table 6. Although four stable clones (7-111, 7-55, 7-47, and 7-109) remained stable, of the four other stable clones that became unstable three were more unstable in the set that was fed twice daily than

TABLE 6

Effect of changing culture fluid daily and twice daily on stability

Clone	Initial serotype	Percent subclones of initial serotype			Percent subclones of new serotypes		
		Beginning* of experiment	Fed daily		Beginning* of experiment	Fed daily	
			Once	Twice		Once	Twice
7-111	C	100	100	100	3	6	13
7-55	G	97	100	98	13	0	0
7-47	G	90	100	96	7	4	4
7-109	X	95	92	91	7	28	26
7-126	G	97	90	35	24	49	92
7-128	G	93	100	93	34	15	34
7-124	G	88	95	100	26	5	33
7-4	X	90	100	87	26	38	13
7-121	G	76	100	98	21	16	16
7-106	X	66	58	73	95	42	49
7-110	G	57	89	94	6	31	2
7-54	X	51	46	80	61	76	46
7-126-1	X	37	70	81	78	55	25

* This column refers to the results of serotype tests of subclones isolated on the first day of the onset of daily feedings. All clones initially were 90-100% of one serotype; the changes recorded in the table refer to subclones originating from these clones.

in the set fed only once daily (as indicated by the percent subclones with new serotypes, an inverse measure of the purity of clones for cells of the initial serotype). All unstable clones became more stable. Of the five unstable clones, in all cases but one (7-121) the clones in the set that was fed twice daily were more stable (as measured by the percent subclones having cells of the initial serotype) than their sister clones fed once daily.

A similar experiment was carried out with a few cultures maintained at 17° (Table 7). Because of the slower growth rates feedings at this temperature were at 24-hr or 48-hr intervals. All of these were unstable clones and at this temperature the % subclones of the initial serotype were little affected by any of the growth conditions. However, the proportion of subclones pure for the initial serotype ("% subclones 100% of initial serotype") was often markedly different among those from the set repeatedly fed and those from the original culture. Once

TABLE 7

Effect of changing culture fluid every 48 and 24 hours on stability (17° set)

Clone	Initial serotype	Percent subclones of initial serotype			Percent subclones 100% of initial serotype		
		Original	Fed every		Original	Fed every	
			48 hr	24 hr		48 hr	24 hr
7-7	G	92	70	87	77	8	23
7-24	X	81	88	93	79	79	91
7-121	G	77	96	84	24	15	24
7-123	G	78	90	82	33	8	24
7-124	G	88	84	93	68	20	30

again, the tubes from which the old medium was removed most often yielded the more nearly stable condition.

A second, entirely distinct approach was also used to determine whether the transformations between stability and instability brought about by a change of culture fluid were due to removal of the "old" medium. It was reasoned that in a short-term experiment a nutrient-poor medium (indeed a salt solution), rather than a nutrient-rich medium, should serve as a replacement for the original exhausted medium. Obviously, since conversion of the stability character of a culture requires an extended period of time, conversion could not be followed directly. Instead, a related effect of replacing medium was examined. As noted in MATERIALS AND METHODS, the proportion of subclones with cells of a particular serotype may be larger when these subclones are isolated from a sample of a clone kept overnight in fresh culture fluid than when isolated from a sample of the same clone allowed to remain in its own medium. When a clone behaves in this fashion, another set of samples—taken the following day from the original culture—will yield subclones (again isolated after overnight incubation) having this same serotype in greater abundance than did the subclones from the first set of samples. Thus, the action of fresh culture fluid on a sample of a culture is an indicator of events occurring in the original culture. The fact that these events have to do with the distribution of serotypes among subclones from a culture with a uniform serotype is, in a general sense, not dissimilar from the extreme distribution which we call stability and instability.

Table 8 presents the results of four representative experiments in which a culture was divided into three aliquots. To one aliquot was added an equal volume

TABLE 8

Comparison of the effects of exhausted medium, fresh culture fluid, and a salt solution (Dryl's solution) on unstable clones

	Total tested	Number of subclones with serotype			Percent subclones with serotype		
		G	C	X	G	C	X
Clone 7-23							
Fresh culture fluid	75	9	2	67	12	3	89
Own exhausted medium	78	11	4	59	14	5	76
Dryl's solution	74	7	3	71	9	4	96
Clone 7-34							
Fresh culture fluid	77	70	6	34	90	8	44
Own exhausted medium	82	72	2	26	88	2	32
Dryl's solution	77	72	1	38	94	1	49
Clone 7-46							
Fresh culture fluid	36	4	34	10	11	94	27
Own exhausted medium	52	12	36	10	23	69	19
Dryl's solution	46	2	44	7	4	96	15
Clone 7-32							
Fresh culture fluid	40	24	22	2	60	55	5
Own exhausted medium	48	46	6	7	96	13	15
Dryl's solution	53	27	32	6	51	60	11

TABLE 9

Comparison of effects of overnight incubation in fresh culture fluid and in Dryl's salt solution

Sign of Δ between fresh culture fluid and exhausted medium	Number of clones in which the effect of Dryl and fresh culture fluid were		Total number of clones
	Same	Different	
+	17	4	21
-	22	3	25
0	69	9	78
Totals	108	16	124

Percent agreement between effects of fresh culture fluid and Dryl's solution = 87.1.

of fresh culture fluid, to a second a nonnutrient medium (Dryl's salt solution), and an equal volume of the original medium was returned to the third. The following day 60 cells were isolated from each aliquot and, after a week's growth in fresh medium, each subclone was tested for serotype. Originally, each aliquot was about 90% of one serotype, with the three samples from the same culture indistinguishable.

From the data in Table 8 it can be seen that generally the serotype that increases in fresh culture fluid is the one that increases in salt solution; the one that decreases in one decreases in the other. In these experiments a shift in one of the serotypes of 10% was considered meaningful. (The exact percentage chosen should not be critical as long as all variables were judged by the same criterion.) The distribution of serotypes among the two sets is not identical, but the directions of transformation are the same. More extended observations fully support this correlation (Table 9).

DISCUSSION

Although the observation that cells of the same serotype nonetheless may differ in the extent to which this serotype will persist among their descendants can have far-reaching implications, of greater interest, and of perhaps more general relevance, is the observation that to demonstrate new serotypes among the progeny of unstable clones cells must be isolated into fresh medium. Because the effect of the medium is not related to its nutritional value but rather is a consequence of its serving as a diluent of the cell's original medium, apparently the significant feature here is the separation of the cell from its neighbors. Since cell to cell contact is not essential for this interaction (FINGER and HELLER unpublished; FINGER 1968), one could conclude that something in the medium affects the stability or instability of a clone.

Previously it had been reported that medium taken from one culture and added to a second could induce changes in serotype among subclones originating from the recipient culture (FINGER, HELLER and LARKIN 1967). A number of "conditions" had to be met for this induction to occur, but the essential phenomenon was plain: the medium apparently contained cell products which could play a controlling role in the synthesis or appearance of serotypes. An hypothesis pro-

posed at this time (FINGER 1967) was that the medium contained a collection of inhibitors or "repressors" of specific antigen synthesis, and the response of a particular clone to added medium was governed by the kinds and amounts of inhibitors the recipient and donor possessed. Stable clones, according to this notion, liberated inhibitors for all antigens except the one expressed. Inhibitors in varying quantities for all antigens, including the one expressed, were to be found in the medium from unstable clones.

One prediction from this model would be that prolonged and continual removal of medium from a stable clone might depress the level of repressor for one of the unexpressed loci so that the antigen specified by this locus could now come into expression. Stable clones might arise by a similar process except that the serotype which would increase in frequency would probably be the one originally expressed since, presumably, the inhibitor for this antigen would be present in the smallest amount and would be the first diluted out. The data reported here are in full accord with this prediction.

Another prediction is based on the comparison of the two sets of subclones isolated from the samples kept overnight in fresh culture fluid and in the original medium. The serotype which appears with greater frequency among the subclones from the latter is the one which tends to disappear with time among the progeny of the parental culture. If one assumes that this is because the antigen is not being made or is being made at a low rate (disregarding for the moment its nonrecognition on the surface at any time), then when the medium is removed, and the inhibitors directed against this antigen with it, this serotype should increase in frequency. The prediction, too, is fulfilled. Indeed, since the majority of unstable clones could be viewed as not maximally synthesizing the antigen detected on the surface, this explanation could also suffice for the conversion of unstable clones to stable clones, as well as accounting for the relatively few exceptions, i.e., those instances where some other serotype increases in frequency.

Regardless of the validity of these suggested mechanisms for the observed effects of diluting cell products, the observations themselves become part of the description of an increasingly complex system. Where originally it was thought that all antigens but one are repressed by a cell, except for the exceptional case involving two specific serotypes (MARGOLIN 1956), it is now known that many clones, which by the immobilization criterion are of any one of several serotypes, can be shown to regularly contain several antigens (FINGER *et al.* 1972). It has also been shown that clones of a single stock identical according to immobilization tests of samples taken from a test tube, can differ markedly in their tendency to produce subclones with changed serotypes.

From these and other observations a host of questions, many of them new, can be framed. For example, what determines whether a clone is persistently unstable or persistently stable? Why does a new antigen arise when an old one disappears? What is the regulatory mechanism that ensures that some—any—surface antigen is always made? What is the relationship between "primary" antigens, those present in all clones, and "secondary" antigens, those found only in unstable

clones? Is the surface of a particular serotype truly composed of just a single antigen, or is there a mosaic undetectable by the techniques used thus far?

Put into a more general context, the questions deal with the control of the synthesis or appearance of similar classes of proteins without any apparent enzymatic function. In other words, should any one of a number of proteins serve equally well the same function, for example as structural elements, what determines that the total amount of the proteins comprising this class remains constant? The role of cell products released externally in the regulation of the activity of genes in neighboring cells is another aspect of these studies that merits further analysis.

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