INCREASE OF MALIGNANT POTENTIAL OF BHK-21 CELLS BY SV40 DNA WITHOUT PERSISTENT NEW ANTIGEN

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The agar suspension culture method,[1, 2] utilizing the BHK-21 cell line of hamster fibroblasts,[3] provides the basis for a quantitative assay of transformation by polyoma deoxyribonucleic acid (DNA).[4, 5] Since transformation of hamster tissue by SV40 has been effected,[6] attempts were made to establish a transformation assay for SV40 DNA in the same system. This paper reports the effects of SV40 DNA on the BHK-21 cell line. While this work was in progress, a report appeared describing transformation of bovine tissue culture cells by SV40 DNA.[7]

Materials and Methods.—Virus: SV40, strain 777[8] ($LLCMK_2$, African green monkey kidney[5], BSC-1_{1-2})[9] was used throughout. Two pools of purified virus, designated A and B, were produced from 1 and 3 liters of crude lysates of BSC-1 cultures, respectively. The purification procedure was the same as that used previously.[10] These pools consisted of the "full" bands obtained after centrifugation in a RbCl preparative density gradient. The final yields of virus obtained were 5×10^{10} and 1.8×10^{12} tissue culture infectious $doses_{50}$ ($TCID_{50}$) from pools A and B, respectively.

DNA extraction: Two methods were used for DNA extraction. DNA was extracted from SV40 pool A with phenol in the presence of phosphate buffer (0.2 *M*, pH 8.0), versene (10^{-3} *M*), and sodium trichloracetate (0.2 *M*, pH 8.0). After extraction, the DNA was dialyzed for 2 days against NaCl 0.55 *M* in Tris buffer (0.05 *M*, pH 8.0). The details of this method have been described.[11] DNA was extracted from pool B by papain and sodium dodecyl sulfate treatment as described in detail elsewhere;[12] the final material consisted of DNA in "0.1 SSC" (0.015 *M* NaCl with 0.0015 *M* Na citrate). Nucleic acid content was determined on the basis of ultraviolet absorption in the DU Beckman spectrophotometer at 260 mμ (0.025 odu = 1 μg/ml). The final yields of DNA obtained were 75 μg (DNA-A) and 750 μg (DNA-B). (See *Note added in proof.*)

One sample of DNA-B (10 μg in 0.05 ml) was treated for 30 min at 36°C with 25 μg DNase 1 (electrophoretically purified, Worthington Biochemical Corp., Freehold, N. J.) in 0.05 ml Tris buffer (0.001 *M*, pH 7.5) containing 5 mM $MgCl_2$. The control was treated with buffer for the same time. An equal volume of 1.1 *M* NaCl in Tris buffer (0.05 *M*, pH 7.5) was then added and both samples were used immediately.

Agar assay: The assay used was essentially that described for polyoma DNA transformation.[4, 5] Confluent or subconfluent cultures of the BHK-21/C13 line[13] (hereafter referred to as C13) of hamster fibroblasts in plastic Petri dishes were shocked with hypertonic saline (0.37 *M*, then 0.55 *M*) in Tris buffer (0.05 *M*, pH 7.5) and then 0.1 or 0.2 ml of DNA solution in 0.55 *M* NaCl was added.[11] Control plates were similarly shocked, and 0.1 or 0.2 ml NaCl (0.55 *M*) was added. After an adsorption period of 30 min at 29°C, the cells were allowed to recover at room temperature for 30 min in Eagle's basal medium with 10% tryptose phosphate broth, 20% fetal calf serum, 2 mM glutamine, and antibiotics (ETC_{20}). The cells from each exposed plate were dispersed with trypsin at this time or after 18 hr incubation, counted, and planted in 0.33% agar over a layer of 0.5% Noble agar.[1, 2] The cultures were held at 36°C in an humidified atmosphere with 5% CO_2. Colony counts were made in 16–18 days. In experiment 1, 1×10^3 and 5×10^2 cells from control and infected monolayers were plated on 8 glass Petri dishes per dilution and stained with Jenner-Giemsa stain 8 days later; the remaining cells were divided into equal parts and planted in agar in 10 plastic Petri dishes. In experiments 2–4, cells were planted at 2 dilutions, 1×10^5 and 5×10^4, in 5 or 6 agar dishes each; since comparable results were obtained at the two dilutions, the results were pooled for presentation here.

Plaque assay: Plaque assays of SV40 DNA were carried out on monolayer cultures of BSC-1 cells shocked and infected as described above. After 18 hr incubation, the fluid was removed from the plates and an agar overlay was added. The details of this assay were the same as for

SV40 virus,[10] with the exception that the overlay contained twice the concentration of vitamins and amino acids and 10% fetal calf serum.

Serologic studies: Fluorescent antibody (FA) and complement fixation (CF) studies were carried out on cell lines established from agar colonies to determine the presence of the SV40 tumor or neoantigen (hereafter referred to as T antigen)[14, 15] and virion antigen. FA tests were done on cells grown on coverslips, the fixation and staining of which have been described.[14] CF tests were done with 10% cell suspensions; 4–8 units of antibody were employed.[15] In CF tests for detection of antibody to the SV40 T and virion antigens, 4 units of antigen were used.

Transplantation: Weanling Golden Syrian hamsters (*Mesocricetus auratus*) were used for transplantation; 10^4 fifth passage cells from each isolated colony were inoculated into each of 8 animals, four intracerebrally and four subcutaneously. The animals were examined 1–2 times each week for the appearance of tumors. Biopsies were performed and 10% tissue suspensions were prepared by techniques previously described.[8] Animals were test-bled at the time of biopsy and every other week thereafter.

Results.—Effect of SV40 DNA on colony formation in agar and on glass: Table 1

TABLE 1

EFFECT OF SV40 DNA ON THE GROWTH OF C13 CELLS IN AGAR

Expt. no.	DNA preparation, μg[a]		Colony Formation in Agar: Total no. cells planted (× 10^6)	Total Colonies: Number	Total Colonies: Colony formation rate (%)	Colonies in Excess of Expected Background[b]: Number	Colonies in Excess of Expected Background[b]: Colony formation rate (%)[d]	T antigen[c]
1	A	3.15	19	1043	0.055	838	0.044	...
		1.75	18	662	0.037	468	0.026	...
		0.87	14	265	0.019	114	0.008	...
	Saline control		15	162	0.011	...	...	...
2	B	1.6	8.5	679	0.080	644	0.076	0.1
	Saline control		9.0	37	0.004	...	...	0[e]
3	B	1.3	7.5	627	0.084	615	0.082	0.1
	Saline control		7.5	12	0.0016	...	...	0
	KB DNA	1.2	8.0	16	0.002	3	0.0004	0
4	B	1.2	7.5	901	0.12	867	0.12	0.08
		1.2 + DNase	7.5	184	0.025	150	0.020	<0.005
	Saline control		6.7	30	0.0045	...	...	0

[a] DNA μg to which the cells planted were exposed; titrations of the SV40 DNA from both preparations A and B revealed that 1 μg of DNA was equivalent to approximately 700 pfu.
[b] Number of colonies − (control rate × number of cells planted).
[c] SV40 T antigen; percentage of cells with fluorescent nuclei when stained with SV40-tumored hamster serum 24 hr after infection with SV40 DNA.
[d] Number of colonies in excess over background/number of cells planted.
[e] 0 = <0.01%.

summarizes the four agar experiments. Only a small proportion of uninfected C13 cells grew into colonies (0.002–0.01%), the lower proportion being found when fewer cells were planted. In contrast, SV40 DNA-infected hamster cells grew to a considerably greater extent, the frequency of growth ranging from 0.05 to 0.12 per cent. The number of colonies decreased as the DNA was diluted (expt. 1); this relationship was not directly proportional throughout the entire range since the number of colonies, in excess of the "background," is lower than expected at the highest dilution. One possible reason for this may be the "auto-feeder" effect present when large numbers of colonies are present. The frequency of growth was higher with DNA-B than with DNA-A, but there was no significant variation between experiments using DNA-B. Differences in the extraction procedures may account for this difference between the two DNA preparations. No significant growth over "background" occurred when C13 cells were exposed to DNA extracted from control KB cells (expt. 3). Treatment of DNA-B with DNase resulted in an 83 per cent reduction in the number of colonies (expt. 4). In experiments 2–4, 5×10^5 cells,

TABLE 2

PLATING EFFICIENCY ON GLASS OF CONTROL AND DNA-INFECTED C13 CELLS

	No. cells plated*	Approximate no. colonies	Mean	Plating efficiency (%)	p†
Control	10^3	166, 210, 205, 132, 185, 219, 187	186.3	19	$t = 4.49$
SV40 DNA-infected	10^3	268, 249, 234, 233, 228, 231, 258, 224	240.6	24	$p < 0.001$
Control	5×10^2	138, 110, 140, 133, 114, 107	123.7	25	$t = 6.11$ $p < 0.001$
SV40 DNA-infected	5×10^2	177, 177, 167, 150, 175, 168	169.0	34	

* All cells were hypertonically shocked and exposed to hypertonic saline with or without DNA for 30 min (see *Methods*).
† p Value obtained by comparison of means by t test.

after trypsinization, were planted in dishes containing coverslips and stained for the SV40 T antigen 24 hr later (Table 1); the percentage of cells with positive nuclei was approximately the same as that of colony formers. These FA results have been reported in detail elsewhere.[12]

In one experiment (expt. 1) the effect of SV40 DNA infection on colony formation on glass was also determined. The DNA-treated cells plated with a greater efficiency (Table 2) and also produced larger colonies (Fig. 1). Since the DNA-infected colonies were larger and frequently overlapped, only approximate colony counts could be made. Control and infected cells and colonies had similar morphologies; the cells exhibited an elongated, fusiform, fibroblastic shape, had parallel orientation, and contained small nuclei with 2–6 nucleoli.

The colonies formed in agar were round, discrete, and had dense central portions. The colonies obtained from cells exposed to DNA did not differ from those on control plates; however, the cells which did not grow into colonies remained as single cells in the latter plates, whereas approximately 20–30 per cent of the SV40 DNA-treated cells underwent several cell divisions and formed small clumps. These were not present in the inoculum since the proportion of clumps was < 1 per cent.

General characteristics of cell lines established from agar colonies: Thirty-three

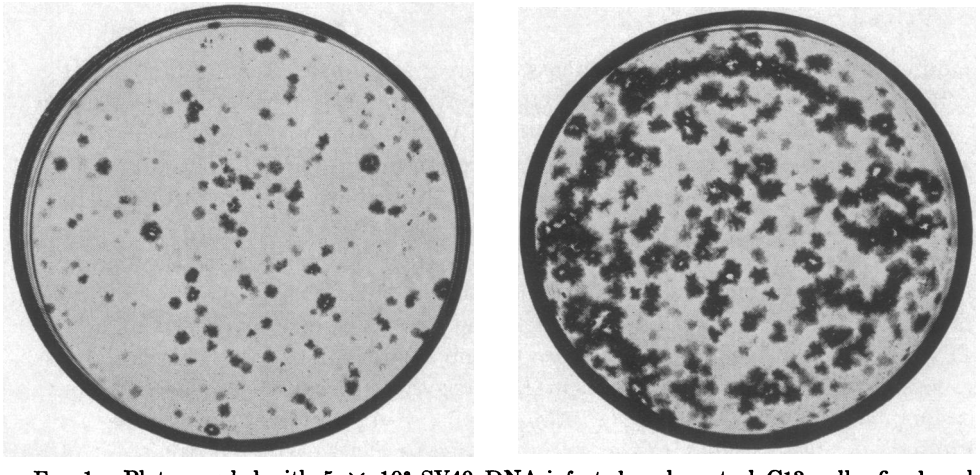

FIG. 1.—Plates seeded with 5×10^2 SV40 DNA-infected and control C13 cells, fixed and stained after 8 days' growth. The plate containing DNA-infected cells, at the right, contains more colonies of larger size than the control plate.

colonies (22 from DNA-treated cells and 11 from controls) from experiments 1 and 2 were picked with fine Pasteur pipettes and grown to mass cultures. These lines were characterized with respect to their morphology, presence of infectious SV40 virus as detected by the cell overlay procedure,[6] presence of SV40 T and viral antigens as determined by FA and CF testing, and plating efficiency in agar. In addition, the cell lines from colonies in experiment 1 were tested for colonial morphology on glass and transplantability in hamsters. Lines from control colonies appeared identical to the parent C13 line, and contained no SV40 virus, T antigen, or viral antigen. With respect to morphology and virus and T antigen content, the lines from DNA-treated cells fell into two distinct categories. Three lines showed SV40 T antigen (but not viral antigen) by both CF (antigen titers of 1:2 and 1:4) and FA (60—80% of cells positive) techniques, and 2 of these 3 lines yielded infectious SV40. Morphologically, the cells of these 3 lines generally were indistinguishable from normal C13 cells, but the cultures contained many multinucleated giant cells of the type characteristic of SV40-transformed cell lines. The other 19 lines from DNA-treated cells were indistinguishable in appearance from control cells and contained no giant cells, SV40 virus, T, or virion antigens. Colonies formed on glass from lines established from DNA-treated and control cells (expt. 1) resembled those from the parent C13 line.

Efficiency of replanting in agar: In order to determine whether the ability of the DNA-infected cells to grow in agar was a stable characteristic and to assess the possible role of selection of pre-existing variants in the C13 population, the cells from all lines were tested for their efficiency of colony formation in agar. Fourth passage cells from all lines were replanted in 0.33 per cent agar in 6 plastic Petri dishes at 10^5 cells per dish. The percentage of cells forming colonies is given in Table 3. Al-

TABLE 3

PLATING EFFICIENCIES IN AGAR OF CELL LINES ESTABLISHED FROM AGAR COLONIES

	Total no. of lines	No. and Percentage of Colonies Having Plating Efficiencies in Given Range				
		0.001–0.009%	0.01–0.09%	0.1–0.9%	1–9%	≥10%
Control	11	1	2	5	3	0
Per cent		9	18	45	27	0
DNA	22	0	0	1	13	8
Per cent		0	0	5	59	36

though the majority of control cells had very low plating efficiencies in agar, most were higher than the parent line (0.007–0.008% for unshocked cells). The lines from DNA-treated cells generally showed much higher plating efficiencies than the controls; some of the lines with efficiencies within the control range may be due to the inclusion of background colonies among those picked from the DNA plates. The 3 T antigen-positive colonies had relatively high plating efficiencies in agar (12, 18, and 26%).

Transplantation to hamsters: The cell lines isolated from experiment 1 were transplanted to hamsters. Tumors appeared in hamsters inoculated with 10^4 cells from 6 of the 7 DNA-infected colonies (Table 4). The subcutaneous tumors were studied more extensively than the intracerebral tumors, since many animals in the latter group died and were eaten or the brains were autolyzed before fixation could be effected. Presumably, these deaths were due to small intracranial tumors.

There was a spectrum of malignant potential among the seven DNA-infected

TABLE 4

TRANSPLANTATION TO HAMSTERS OF CELL LINES ISOLATED FROM AGAR COLONIES (EXPT. 1)

		Subcutaneous Tumors				Intracerebral Tumors	
	Colony	No. tumors/ no. inoc.	Week of appearance	SV40 T antigen in tumors	SV40 T antibody in hamsters[a]	No. dead within 8 weeks[b]	No. tumor demonstrated
DNA-infected	619 #1	4/4	3, 3, 3, 3	0/4[c]	0/4	4	1
	619 #2[d]	5/5	3, 3, 3, 3, 4	5/5	3/5[e]	4	2
	620 #1	3/4	3, 5, 6	0/3	0/3	4	4
	625 #1	4/4	3, 3, 5, 5	0/4	0/4	3	1
	625 #2	0/4	—	—	—	0	0
	625 #3	1/4	7	0/1	0/1	0	0
	625 #4	1/4	5	0/1	0/1	0	0
Control	C #1	0/4	—	—	—	0	0
	C #2	0/4	—	—	—	0	0
	C #3	0/4	—	—	—	0	0
	C #4	0/4	—	—	—	0	0
	C #5	0/3	—	—	—	0	0

[a] These determinations were carried out with 2–6 serum specimens obtained from each tumored hamster; the size of the tumors ranged from 2 to 7 cm (mean tumor diameter) at the time the serum was obtained.
[b] Hamsters with obvious tumors were sacrificed to obtain tumor specimens.
[c] Antigen determinations were made once for each tumor.
[d] Line 619 #2 contained SV40 virus and T antigen. The 6 other lines were virus- and antigen-negative.
[e] Titers ranged from 1:10 to 1:160 according to size of tumor.[15]

lines; two formed tumors in only one of eight animals and one did not transplant at all; however, the cells from four lines formed tumors after short latent periods in practically all inoculated animals. The number of cells required for tumor formation by subcutaneous inoculation was determined for two cell lines (619 #2, 625 #1) and the 50 per cent end points were found to be the same (10^3 cells). No tumors appeared in animals inoculated either subcutaneously or intracerebrally with 10^4 control cells over a 16-week period of observation.

Suspensions of tumors from five of six DNA infected lines were negative for the SV40 T antigen in CF tests; all tumors from line 619 #2 contained this antigen. Antibody to this antigen was demonstrated in the serum of three tumor-bearing hamsters inoculated with this line; no antitumor antibody was detected in 2–6 serial serum specimens from the other tumor-bearing hamsters when tested against standard SV40 T antigen. Moreover, no CF reaction was observed in the latter group when each tumor-bearing hamster serum was tested against the homologous tumor. No antiviral CF antibody was found in the serum from any of the hamsters.

The subcutaneous tumors were soft and had large, necrotic, friable centers; grossly, there did not seem to be a tendency to invade the underlying musculature. However, upon microscopic examination, infiltration of adjacent muscle bundles was occasionally seen. Some tumors which arose after intracerebral inoculation of cells grew extracranially; the remainder either grew in the substance of the brain or in the extradural space. Histologically, all tumors were fairly well differentiated fibrosarcomata. Tumors rising after transplantation of cells from line 619 #2 had many multinucleated giant cells; however, some tumors from line 620 #1 also contained multinucleated giant cells. It is noteworthy that tumors derived from BHK-21 cells which had undergone spontaneous malignant change frequently contained multinucleated giant cells.[16]

The growth of the tumors was progressive, leading to the death of the animal. Subcutaneous tumors often reached sizes of 5–7 cm (mean tumor diameter) before death occurred. The tumors transplanted readily; four have been maintained for 5–9 serial passages thus far (619 #2, 619 #1, 620 #1, 625 #1). No SV40 T antigen

has been found in tumors initiated from the latter 3 lines while tumors from 619 #2 have 4–8 units of SV40 T antigen through at least the 8th transplant passage.

Discussion.—The data reported here show that SV40 DNA produced a "growth stimulus" in C13 cells; the DNA-infected cells plated in agar with 5–50-fold greater efficiency than control cells, and cell lines derived from the DNA-exposed agar colonies showed generally greater replating efficiencies and transplantability to hamsters. Also, the cells exposed to SV40 DNA plated with greater efficiency and formed larger colonies on glass than did control cells.

This stimulation is reminiscent of the "sparing effect" noted in cultures of rabbit, mouse, and porcine kidney which were infected with SV40 virus; cultures which did not undergo transformation survived much longer than uninfected control cultures.[17]

In contrast to most viral transformation systems, the changes observed here must be considered as primarily quantitative rather than qualitative alterations. First, in respect to the properties where a qualitative difference might be found, i.e., cellular and colonial morphology, none was observed, in contrast to transformation of C13 cells by polyoma virus or its DNA. Second, the quantitative differences between DNA-treated and control cell lines were essentially magnifications of differences between cells in the parent line. Thus, plating of control cells in agar resulted in selection of cell lines with generally higher replating efficiency than the parent line, but lower than that of cells from DNA-treated cultures. Also, there is an inherent malignant potential in BHK-21 cells[3] and, to a lesser extent, in the C13 clonal line;[18] Gotlieb-Stematsky and Shilo showed that malignant variants could be selected from both C13 and the parent BHK-21 line by altering environmental conditions.[18]

The displacement of this spectrum of growth potential of C13 cells following exposure to SV40 DNA could represent a selective facilitation of the growth of the more malignant C13 cells, acceleration of a pre-existing process leading to emergence of malignant variants in the C13 line, or a change specifically produced by SV40 but presently recognizable only by quantitative studies. In any case, it is clear that the SV40 DNA did not produce selection by exerting widespread cytolytic or inhibitory effects, since the DNA-treated cells plated with higher efficiency than controls, and the plating efficiency on glass was quite high.

It is unlikely that the effects described here are nonspecific effects of any DNA. KB cell DNA had no effect on C13 cells, and in another laboratory attempts to stimulate growth of C13 cells at approximately the same passage level with DNA extracted from normal and polyoma-transformed C13 cells[19] and DNA extracted from human papilloma virus[20] have failed.

The majority of cell lines from the DNA-treated cultures did not demonstrate SV40 T antigen, which heretofore has been found in all SV40 virus-transformed cell lines. However, since the number of cells forming colonies was essentially identical to the number containing T antigen 24 hr after exposure to SV40 DNA, and since T antigen generally does not persist in C13 cells,[12] it appears possible that the antigen-negative colonies were formed from cells which had transitorily produced the antigen. In this connection it may be significant that the T antigen-positive clonal lines did not demonstrate antigen in all cells as do the SV40 virus-transformed cell lines;[14] these lines did not appear to be losing the antigen, since about the same percentage of cells were positive throughout the 4th–12th passages. This may in-

dicate loss of antigen from one or more daughter cells in the earliest stages of colony development. The lines which contained T antigen were among those with the highest replating efficiencies in agar, and in the case of the one line tested, in ability to transplant into hamsters, but they were not outside the range of the antigen-negative lines in these respects. Additional attempts to detect persistent SV40 genetic material in the antigen-negative lines are in progress, including transplant rejection and nucleic acid hybridization techniques.

Comparisons with the effects of polyoma DNA on C13 cells reveal many similarities[4, 5] in the quantitative aspects of colonial growth in agar; the main difference is that infection of this cell line with polyoma DNA results in a morphological transformation, whereas colonies resulting from SV40 DNA infection were composed of cells with normal morphology. One μg of polyoma DNA resulted in 200–1500 transformations, while 300–700 colonies resulted from 1 μg of SV40 DNA; 2–15 pfu and 10^8–10^9 molecules of polyoma DNA were required for one transformation, while 1–3 pfu and 3–7 $\times$ 10^8 molecules of SV40 DNA (mol. wt. 3 $\times$ 10^6)[21, 22] were required for the production of one colony. Comparisons with the effects of whole virus on this cell line show marked differences. Polyoma virus causes a morphological transformation in 5–10 per cent of C13 cells,[23] whereas exposure of this cell line to SV40 virus at virus:cell multiplicities of as high as 10^3 results in no morphological transformation and less growth stimulation than obtained here with DNA.[24] This may be due to the very poor adsorption of SV40 virus to C13 cells,[24] whereas infection with SV40 DNA bypasses the adsorption block.

Summary.—BHK-21/C13 hamster fibroblasts exposed to SV40 DNA plated with greater efficiency in agar and on glass than control cells. Cell lines established from the agar colonies from DNA-treated cells were morphologically normal but replated in agar with higher efficiency than cell lines from control colonies, and 6 of 7 produced tumors on transplantation into hamsters. Only 3 of the 22 cell lines from DNA-treated cell colonies contained SV40 T antigen; lines which did not contain the T antigen transplanted to hamsters as readily as an antigen positive line. Cell lines from control colonies replated in agar with higher efficiency than the parent line, but did not produce tumors in hamsters.

The results indicate that SV40 DNA produced an increase in the growth potential of BHK-21/C13 cells which may represent an effect different from that in the usual viral transformation systems.

Note added in proof: Drs. Lionel Crawford and Kurt Cohn kindly analyzed DNA preparations A and B, respectively, using the band centrifugation method of Vinograd.[25] Ultraviolet absorption photographs were taken every 32 min and the amounts and sedimentation coefficients of the DNA components estimated from the microdensitometer tracings obtained. DNA-A was composed of 90 and 10% in the fast (21.2*S*) and slow (16.1*S*) forms, respectively, while DNA-B was composed of 27 and 73% in the fast and slow forms, respectively.

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*MECHANISM OF ACTION OF PENICILLINS: A PROPOSAL BASED ON THEIR STRUCTURAL SIMILARITY TO ACYL-D-ALANYL-D-ALANINE**

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The mechanism by which penicillins kill bacterial cells has been of interest since their discovery in 1929.[1] In retrospect, three important observations stand out from the early studies. In general, penicillins kill gram-positive bacteria more effectively than gram-negative bacteria.[2] They kill rapidly dividing cells but not resting cells,[3] and induce morphological alterations in treated cultures.[4, 5] As early as 1946 it was suggested that the formation of bizarre and filamentous forms could be accounted for by the loss of integrity of the cell wall.[5]

Subsequently, cell walls were isolated and examined.[6] The walls of gram-positive bacteria have a remarkably simple composition compared to those of gram-negative organisms, a major difference between the two groups being the occurrence of lipid in the latter. Lysozyme[7] is a glycosidase which kills sensitive bacteria by solubilizing the cell wall.[8] In hypertonic sucrose broths, sensitive bacilli are not lysed but are converted to spherical forms termed protoplasts.[9] Thus, the wall determines the shape of bacteria and is responsible for their stability.