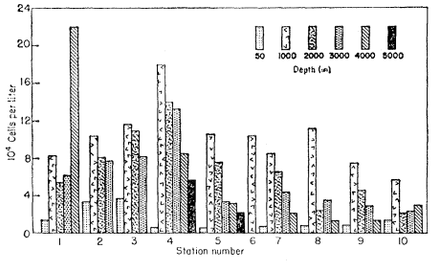

Fig. 3. Abundance of flagellates in tens of thousands of cells per liter observed on "Sweat" cruise.

Casbah cell counts are not complete yet, those completed are of a magnitude similar to those of Sweat. Although these data were collected with nonsterile samplers, their order of magnitude has been corroborated by the use of the previously mentioned Cobet samplers. The vertical distribution (Fig. 3) indicates a relative paucity in the euphotic zone (50 m averaged 15,000 cells/liter), followed by maximum numbers in almost every case at 1000 m (average, 107,000 cells/liter). Below 1000 m, a gradual decline occurs, yielding numbers at 5000 m (average, 39,000 cells/liter) almost three times those found near the surface. It is interesting to note that the maximum number (220,000 cells/liter) observed for Sweat occurred at 4000 m at station No. 1. A horizontal variability also appears to exist, indicating either a decline with approach to land, that is, at both ends of the transect, or possibly an increase in numbers with approach to that area most influenced by the Amazon discharge. It is hoped that the completed Casbah data will help to clear up some of the reasons for this variability.

Rates of glucose and acetate uptake determined during the Casbah cruise, although quite variable, suggest that this deep community may utilize dissolved organic substances. Although substantial numbers of organisms were present in each case, and shipboard techniques were sterile, some of the observed uptake is open to question because of the lack of sterility in the samplers. These experiments will soon be reproduced again at sea and in the laboratory with axenic cultures.

It would appear that these unicellular, motile, microorganisms are ideally suited for this aphotic environment where diffusion of organics in low concentration is so essential. Their small size results in a high area-to-volume ratio for increased efficiency in uptake, while their motility allows them to maintain a high internal/external gradient across external membranes. It appears that these results support Bernard's contention of a heterotrophic existence for his Mediterranean coccolithophores, since the conditions for existence of both are quite similar.

Thus, these results suggest that a fairly abundant phytoplankton population, possibly capable of heterotrophic existence, exists at a depth between 1000 and 5000 m, between latitudes 40°N and 3°S in the Atlantic Ocean.

The reason these organisms have seldom been observed seems to rest with the method of sample treatment and observation, namely, either centrifugation prior to examination, or sedimentation with the inverted microscope. The former method was used by the expeditions (*1*) mentioned earlier, but has since been shown to be quite inefficient with certain organisms (*10*). The inverted microscope is also inadequate for these small flagellates, although Bernard has used it successfully in the enumeration of coccolithophores (*4*, *10*). A comparative study made with identical Sweat samples demonstrated that after 48 hours most of the flagellate cells (Fig. 2) could still be recovered by filtering the supernatant from the settling chambers. This means that after fixation these cells either require a very long time to settle out or do not settle out at all. Therefore, it appears that both settling and centrifugation are methods which are inadequate to detect these organisms.

The implications of a community, whose presence has been generally unknown, which is both abundant and apparently autochthonous throughout the aphotic zone of the North Atlantic would appear to be far-reaching; for the presence of an actively metabolizing flora with cell numbers 100 times greater than has been previously reported (*1*) has general oceanographic importance. To mention several areas possibly influenced by the knowledge of the presence of these organisms, we should include: present estimates of recycling rates of organic matter in the sea; the nutrition of herbivorous deep zooplankton communities; and the question of the apparent paucity of bacteria in the deep sea where organic material is available and other organisms are abundant. In any case, the presence of these organisms presents an important area of investigation which biological oceanographers have long overlooked.

ROBERT O. FOURNIER
Graduate School of Oceanography, University of Rhode Island, Kingston

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11. I thank Theodore J. Smayda, under whose supervision this study was undertaken and whose assistance has been invaluable; Drs. David M. Pratt and John McN. Sieburth for advice throughout; and Prof. Francis Bernard, who aided me with many identifications. This work was supported in part by NIH training grant ES-41-02 and NSF contract GB-1500, and is in partial fulfillment of the requirements of the degree of doctor of philosophy from the University of Rhode Island.

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Susceptibility of Human Diploid Fibroblast Strains to Transformation by SV40 Virus

Abstract. *A quantitative system has been developed for the study of transformation of human diploid fibroblasts in culture by two oncogenic viruses, SV40 and the E46 strain of adeno 7-SV40 "hybrid" virus. Seven of the eleven cell strains derived from human skin biopsies when infected with SV40 (10^9 tissue culture infective doses per milliliter) gave rise to transformed colonies with approximately the same frequency (0.03 percent). Two strains derived from patients with Fanconi's anemia, an autosomal recessive disease associated with a high incidence of chromosome abnormalities and spontaneous neoplasms, gave values more than ten times higher. Two strains from persons heterozygous for this gene were also considerably more susceptible to viral transformation.*

Several quantitative systems for studying the in vitro transformation of cells in tissue culture by oncogenic viruses have been described; these have employed both diploid fibroblast strains

(*1*) and established fibroblast lines (*2*) from various infrahuman species. Human diploid cells are transformed by SV40 virus (*3*) and by an adeno 7-SV40 "hybrid" virus in mass culture (*4*), but the frequency of these transformations has not been studied quantitatively. By using sufficiently high titers of virus and inoculating the virus-infected cells at the proper cell density it is possible to measure the frequency of virus-induced transformation of human diploid cell strains. The values obtained from normal cells fall into a narrow range, while an abnormally high susceptibility to transformation is seen in cell strains derived from certain individuals.

Eleven different cell strains were initiated from skin biopsies from both adults and children. Seven of these were obtained from normal persons or from hospital patients with diseases not associated with an increased incidence of malignancy. Two strains (A.M., 35-year old female; J.V., 7-year-old male) came from patients with Fanconi's anemia. This disease is characterized by progressive pancytopenia appearing in an individual with various congenital malformations (*5*, *6*). The disorder is inherited in autosomal recessive pattern; there are no clinical manifestations in the heterozygotes except that, as in the homozygous condition (*6*), there is an increased incidence of neoplastic disease (*7*). Two biopsies were taken from obligatory heterozygotes: C.V., the 39-year-old mother of J.V., and T.M., the 13-year-old daughter of A.M. All the cell strains were tested for their sensitivity to transformation between the third and eighth subculture in vitro.

The viruses used were SV40 strain 776, concentrated tenfold by centrifugation, and titering $10^{9.0}$ tissue culture infective doses (TCID) per milliliter on African green monkey kidney cells, and the E46 strain of adeno 7-SV40 hybrid virus (*8*), titering $10^{8.2}$ TCID/ml on human embryonic kidney cells. Infection was performed by adding 0.5 ml of the virus preparation to a petri dish containing 2 to 5×10^5 exponentially growing cells. The plate was tilted back and forth every 15 minutes for 3 hours, the cell layer was washed five times with serum-free medium, and then complete medium was added.

From this time on the cells that were infected with SV40 were maintained in medium supplemented with 0.5 percent rabbit anti-SV40 antiserum (neutralization titer of 1024) and the cells infected with the hybrid virus were kept in the presence of 0.5 percent rabbit anti-adenovirus type 7 antiserum (neutralization titer of 512). On the day after infection, the cells were transferred to 12 fresh 50-mm petri dishes at densities of from 1×10^4 to 5×10^4 cells. Counts of attached cells 4 and 24 hours later indicated that a fraction of the cells were already killed by the virus. The proportion was about the same for each of the eleven cell strains: less than 20 percent for SV40, but more than 60 percent for the E46. Continued cell destruction was effectively prevented by the appropriate antiviral serum. The medium (Dulbecco and Vogt, containing 10 percent calf serum) was changed twice weekly, and 16 to 20 days after plating the cultures were fixed with 10 percent formalin in phosphate-buffered saline and stained for 10 minutes with 1 percent hematoxylin.

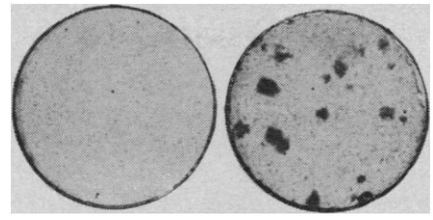

Fig. 1. Cultures of human diploid cell strain HG fixed and stained 17 days after inoculation of 5×10^4 cells. (Left) Control. (Right) Culture which had been infected with $10^{9.0}$ TCID/ml of SV40 1 day prior to plating.

The inoculated cells, both control and infected, formed a confluent monolayer within 8 to 10 days. Transformed colonies could be identified against the background of untransformed cells by their dense and disordered pattern of growth. Such regions stained more darkly than the untransformed areas and were recognized grossly. No dark-staining transformed colonies were ever seen in the uninfected cultures. Figure 1 shows an uninfected culture of strain HG and a culture infected 18 days previously with SV40. The control cells grew into a confluent, rather homogeneous sheet of cells, while the infected cells developed several discrete, dark-staining foci of transformed cells. Such colonies can also be recognized in the living state, isolated, and the cells transferred and serially propagated. The transformants produced both by SV40 and E46, when grown up into mass culture, have been shown to contain the SV40 specific T antigen (*4*).

The inoculation density of the virus-infected cells is critical for the assay; if they are overdiluted (fewer than 10^4 cells per plate) their colony-forming ability falls off sharply. If they are inoculated at a cell density greater than 1×10^5 per plate they go through too few cell generations before becoming arrested by contact inhibition, and as a result the transformed colonies fail to become expressed. This requirement, that several cell generations elapse subsequent to infection for the transformed character to develop, was first shown for SV40 infected cells of the mouse line 3T3 (*9*). Except for the need for antiviral serum to arrest the destruction of the cells by the virus, the assay is essentially the same as that previously described for polyoma virus and rat fibroblasts by Williams and Till (see *1*), and for SV40 and mouse cell line 3T3 by Todaro and Green (see *2*).

The results in Table 1 show that the seven cell strains derived from normal persons and from individuals with disorders not associated with an increased incidence of malignancy gave values for transformation frequency by SV40 of 1.6 to 5.1 per 10^4 cells. The other four cell strains gave considerably higher values. The two strains from patients with Fanconi's anemia, A.M. and J.V., gave values of 79.7 and 41.4 per 10^4 cells, while the heterozygous strains, T.M. and C.V., gave somewhat lower values, 20.1 and 28.2. These data suggest that the heterozygous cells, while clearly out of the normal range, are not quite as susceptible as the homozygous cells, but a more detailed study would be needed to confirm this.

Five of the eleven cell strains, four normals and strain A.M., were also tested for their susceptibility to transformation by the adeno 7-SV40 hybrid virus strain E46. Table 1 shows that with this virus too, cell strain A.M. was at least 20 times more susceptible to viral transformation than the other four cell strains. Direct comparison of the transforming efficiency of SV40 and E46 is not possible from the data presented because different virus concentrations were used and because the cell-killing by the "hybrid" virus was more extensive.

Table 1. Transformation frequency of virus-infected human cell strains. Values are means, ± standard errors, of two to six separate experiments in each of which a total of at least 2×10^5 infected cells were plated.

Cell strain	Transformed colonies / cells plated $\times 10^4$	
	SV40*	E46†
A	4.4 ± 1.1	0.8 ± 0.3
PB	1.6 ± 0.2	
JW	4.3 ± 0.6	0.5 ± 0.2
LG	5.1 ± 0.6	0.4 ± 0.3
HG	2.6 ± 0.7	0.4 ± 0.2
LK	3.0 ± 0.5	
RT	3.7 ± 0.6	
Fanconi's anemia, homozygous		
AM	79.7 ± 18.1	16.3 ± 3.4
JV	41.4 ± 12.5	
Fanconi's anemia, heterozygous		
TM	20.1 ± 3.2	
CV	28.2 ± 8.7	

* Infected with $10^{9.0}$ TCID/ml. † Infected with $10^{8.2}$ TCID/ml.

Since the probability of a cell-virus interaction leading to transformation of human diploid cells is extremely low, high titers of infecting virus are required to produce a sufficient number of transformants for scoring. However, once initiated, the transformation proceeds rapidly and colonies of transformed cells can be seen within 10 days of infection. When conditions different from those described here were employed, and lower virus titers used, transformed cells could not be detected until several weeks after infection (*10*).

The other quantitative system that is available for studying the in vitro transforming effect of SV40 utilizes the mouse cell line 3T3; with this system one transforming unit of virus corresponds to roughly 10^3 infectious units and to 10^5 physical particles (*11*). From the present results it appears that the transformation by SV40 of human diploid cells is about 300 times less efficient than in the case of 3T3; a virus concentration ($10^{9.0}$ TCID/ml) that will transform roughly 10 percent of a 3T3 population, transforms about 0.03 percent of the normal human diploid cells.

Congenital malformations and a progressive pancytopenia are seen in the homozygous individuals in Fanconi's anemia, while their heterozygous offspring and parents do not have these abnormalities. There is a high incidence of leukemias and solid tumors (*6*) in the homozygous individuals, and of leukemias in their relatives (*7*). Cells from two obligatory heterozygous carriers of the gene as well as cells from two homozygotes have a high susceptibility to malignant transformation in culture. Probably this assay can be used to identify nonobligatory heterozygotes in an affected family. The increased risk of neoplasia associated with this disease may be the result of a greater cellular susceptibility to transformation in vivo.

Cells of the hamster line BHK21 and the mouse line 3T3 have been rendered more susceptible to viral transformation by treating them, respectively, with x-irradiation (*12*) or with the thymidine analog, bromodeoxyuridine (*13*), in sublethal quantities prior to infection with the oncogenic virus. In these systems the thymidine analogs and the x-irradiation do not, in themselves, produce cellular transformation, but they potentiate transformation by the virus. Both bromodeoxyuridine and x-irradiation are also known to produce chromosomal damage (*14*).

In Fanconi's anemia, the frequency both of chromosomal aberrations (*6*) and of the viral transformation is increased; but in this case, too, it is not clear that the enhanced transformation frequency depends upon the increased chromosome breakage and reorganization. Transformation by oncogenic DNA viruses may well require the interaction of a portion of the viral genome with that of the host cell (*9, 15*), and cellular replication errors due either to external agents or genetic defects may favor transformation by facilitating this interaction.

The use of human diploid fibroblasts for the study of transformation by tumor viruses has advantages over systems using diploid cells from other species. Human fibroblasts, unlike those of most other species, do not spontaneously "transform" into cell lines (*16*). In this system transformed clones can be detected and isolated quite early; this might permit a reexamination of the relation of chromosomal alterations to the transformation process (*17*). With this assay it is also possible to measure directly the oncogenic potential of various viruses for human cells. For example, it would be of interest to compare the transformation frequency produced in human diploid fibroblasts by the various adeno-SV40 "hybrid" viruses with their relative oncogenicity in the hamster (*18*).

Finally, one can study cell strains from patients with other diseases associated with an increased incidence of neoplasia, such as Bloom's syndrome (*19*) and Down's syndrome (*20*). Cells from patients who have already developed cancer might also show an unusual susceptibility to transformation in culture by tumor viruses.

GEORGE J. TODARO
HOWARD GREEN
Department of Pathology, New York University School of Medicine

MICHAEL R. SWIFT
Department of Medicine, New York University School of Medicine, New York 10016

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