ment 4: Since in the above experiments the test solution contained 20% HT-NRS, it was of interest to determine whether or not HT-NRS was essential for the chemotactic activity of the lysosome lysate. In this experiment (Table II), HT-NRS was omitted from the test solution, but no significant differences between the 2 test solutions were observed.

Discussion. This study has shown that leukocytes are able to reverse their direction of migration in response to an oppositely applied concentration gradient of chemotactic serum. This observation indicates that the active serum factor does not act irreversibly, and that the cells are continuously responsive to a gradient of active substance in this *in vitro* test.

The second part of the study has indicated that there is some chemotactic factor associated with the lysosomal fraction of rabbit leukocytes which does not require fresh serum for its activity. Ward *et al*(3) have reported in an addendum that neither lysosomal lysate nor the anti-inflammatory polycationic protein showed chemotactic activity *in vitro*. However, it is felt that differences in the assay of chemotaxis, particularly in the pore size of the filter employed, could be responsible for these conflicting results. Under the conditions employed in this laboratory, the lysosome-related factor induces less migration than the serum factor. It is not clear whether this is a result of concentration differences, or whether a more complex mechanism acts *in vivo* to produce the marked inflammation observed by Janoff and Zweifach(5).

Even though chemotactic activity has been associated with lysosomes, the possibility must be considered that the leukocytes ingested the active serum factor during glycogen stimulation *in vivo* and that it was localized in the lysosomal portion after fractionation of the harvested cells. All preparations of granules were obtained from cells which had been stimulated with glycogen.

Summary. Using Boyden's *in vitro* method, it was demonstrated that rabbit polymorphonuclear leukocytes reversed their direction of migration in response to a concentration gradient of chemotactic serum. The presence of a chemotactic factor or factors associated with the lysosomal fraction of leukocytes which does not require fresh serum for its activity was shown.

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Calcium Sensitivity of Cell Cultures Derived from Adenovirus-Induced Tumors.* (31264)

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Certain strains of human adenoviruses have been shown to induce tumors in hamsters(1), rats(2) and mice(3). Cells from these tumors were transplantable(2) and contained virus-specific complement-fixing and fluorescent

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antibody tumor cell antigens(2,4). Although cultures derived from adenovirus-induced tumors have been passaged serially *in vitro*(5), they were often difficult to grow, and in monolayer the colonial morphology was often characterized by formation of ball-like aggregates or clumps(6). In the case of 2 cell lines derived from type 12 adenovirus-induced hamster tumors, the clumped morphology and poor growth characteristics were shown to be associated with a cellular sensitivity to calcium at concentrations usually used for tissue culture media(7).

The present study was undertaken to determine the range of cell lines sensitive to calcium. The results suggested that various degrees of calcium sensitivity were not limited to type 12 adenovirus-induced tumor cell lines, but were characteristic of cell lines derived from tumors induced, or cell lines transformed *in vitro,* by adenovirus types 3, 7, 12 and 18. Further, since cell lines derived from other virus-induced tumors and standard heteroploid and diploid cell lines were not sensitive to calcium, calcium sensitivity appeared to be useful as a marker to help determine whether a tumor was adenovirus-induced.

Materials and methods. Cell lines transformed by adenoviruses or derived from adenovirus-induced tumors routinely were maintained in Eagle's Minimum Essential Medium formulated without calcium but supplemented with 0.1 mM calcium chloride, 0.1 mM nonessential amino acids, 5% dialyzed calf serum and 1% fetal calf serum. All other cultures were maintained in Eagle's Minimum Essential Medium (1.8 mM calcium chloride) supplemented with 5-10% normal calf serum. Cultures to be tested were prepared in replicate and, when confluent growth was achieved, refed with media containing 0.1 mM, 2 mM, 5 mM and 7.5 calcium chloride, at a pH of 7.2, and examined daily for 4 days under double-blind code. A cell line was considered calcium sensitive when the colonial morphology at 7.5 mM calcium showed retraction or clumping in comparison with the 0.1 mM control.

To quantitate the optimal calcium concentration for a cell line, long-term serially propagated cultures were carried in calcium-free media supplemented with various concentrations of calcium and 5% dialyzed calf serum.

Results. As shown in Table IA, calcium sensitivity tests were run on 39 cell lines derived from *in vivo*-induced tumors. Each of the 22 calcium-sensitive lines was derived from a hamster tumor associated historically with adenovirus infection. These calcium-sensitive or positive cell lines were derived from tumors induced by adenovirus types 3, 7, 12 and 18, or by an adenovirus 3-SV_{40} (Morris, J. A., *et al.,* personal communication), adenovirus 7-SV_{40}(8) or an adeno 12-SV_{40}(9) "hybrid" virus, or by simian virus 20 (Morris, J. A., *et al.,* personal communication). In contrast, 15 of the 17 calcium negative cultures were derived from non-adenovirus-induced hamster tumors, including tumors induced by Schmidt-Ruppin (Sarma, P. S., *et al,* personal communication), SV_{40} (10), polyoma(11), a chemically-induced hamster tumor(12) and miscellaneous tumors of rabbit, monkey, rat and human origin. One adenovirus type 7-induced tumor line and a "CELO" virus-transformed tumor line were not sensitive to 7.5 mM calcium.

As shown in Table IB, 8 cell lines transformed *in vitro* by virus were also tested for calcium sensitivity. Of the 5 positive lines, 4 were derived from hamster, rabbit and rat cells transformed by adenovirus type 12. Also, one SV_{40}-transformed hamster kidney line was repeatedly calcium-positive. Each of the 3 negative cell lines was not associated historically with an adenovirus infection and included two SV_{40}-transformed hamster kidney cell lines and one bovine papilloma-transformed bovine conjunctiva cell line.

As shown in Table IC, none of the 15 primary, 3 diploid or 7 heteroploid cultures tested showed a tendency to clump in the presence of 7.5 mM calcium chloride. Considering all of the data together, 25 of 27 calcium-positive and 2 of 45 calcium-negative cell lines were associated historically with an adenovirus infection.

Most of the cell lines derived as a result of adenovirus induction or transformation, when tested against the appropriate sera from tumored animals, contained demonstrable complement fixing tumor antigens but several

calcium-positive cell lines did not contain demonstrable tumor antigens when tested directly. The antigen negative lines included 2 adenovirus 12-transformed rat lines supplied by Levinthal(13) and 2 adenovirus 7-SV_{40}-induced hamster tumor lines (herein desig-

TABLE I. Calcium Sensitivity of Cell Cultures.*

Type	Group	Associated virus	No. individual lines tested	No. positive†	No. negative‡
A *In vivo* induced tumors	Adenovirus-induced hamster tumors	Adenovirus type 3	5	5	0
		" type 7	5	4	1
		" type 12	6	6	0
		" type 18	1	1	0
		" 3 - SV_{40} hybrid	1	1	0
		" 7 - SV_{40} hybrid	2	2	0
		" 12 - SV_{40} hybrid	2	2	0
		Simian virus 20	1	1	0
		CELO	1	0	1
		Group totals	24	22	2
	Non-adenovirus-induced hamster tumors	Schmidt-Ruppin	2	0	2
		SV_{40}	2	0	2
		Polyoma	2	0	2
		Fortner fibrosarcoma #3	1	0	1
		Group totals	7	0	7
	Non-adenovirus-induced tumors from animals other than hamsters	Papilloma (rabbit)	1	0	1
		Schmidt-Ruppin (monkey)	1	0	1
		Rauscher (rat)	3	0	3
		Moloney (mouse)	2	0	2
		Bittner (mouse)	1	0	1
		Group totals	8	0	8
B *In vitro* transformed cell lines	Adenovirus-transformed cell lines	Adenovirus type 12—Hamster embryo	1	1	0
		Adenovirus type 12—Rabbit kidney	1	1	0
		Adenovirus type 12—Rat kidney	2	2	0
		Group totals	4	4	0
	Non-adenovirus-transformed cell lines	SV_{40}—Hamster kidney	3	1	2
		Bovine papilloma—Bovine conjunctiva	1	0	1
		Group totals	4	1	3
C Control cell lines	Primary	Hamster embryo	2	0	2
		" kidney	6	0	6
		Rabbit kidney	1	0	1
		Rat kidney	1	0	1
		" embryo	4	0	4
		Mouse spleen & thymus	1	0	1
		Group totals	15	0	15
	Diploid	Human embryonic lung	2	0	2
		Rainbow trout gonad	1	0	1
		Group totals	3	0	3
	Heteroploid	Hamster kidney	1	0	1
		Human, normal	2	0	2
		" carcinoma	2	0	2
		Mouse, normal	1	0	1
		African green monkey kidney	1	0	1
		Group totals	7	0	7

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† Positive = Clumping in presence of 7.5 mM Ca or less.

‡ Negative = No clumping at 7.5 mM Ca.

TABLE II. Differences in Calcium Sensitivity of Adenovirus-Induced Tumor Cell Lines.

Virus	Cell strain No.	Day positive at various calcium concentrations				Long term sensitivity to 1.8 mM Ca
		0.1 mM	2.0 mM	5.0 mM	7.5 mM	
Adenovirus type 3	5913	—*	4	2	2	NT†
	5600	—	2	1	1	NT
	5866	—	—	4	4	NT
	5868	—	—	—	3	NT
	5823	—	1	1	1	NT
" " 7	5916	—	3	1	1	NT
	5914	—	—	—	—	NT
	5766	—	3	1	1	NT
	5739	—	1	1	1	NT
	5728	—	—	—	3	NT
" " 12	5208	—	—	2	2	+, P_6‡
	5512	—	—	3	3	NT
	5524	—	—	3	3	NT
	5125	—	—	2	1	+, P_6
	5204	—	4	3	1	+, P_7
	5209	—	—	3	2	+, P_{10}
" " 18	5207	—	4	2	1	+, P_6
	5116	NT	NT	NT	NT	+, P_5
	5206	NT	NT	NT	NT	+, P_5
Adeno 3 - SV_{40}	H2918	—	—	—	4	NT
" 7 - SV_{40}	H2886	—	—	1	1	NT
	H2266	—	—	1	1	NT
" 12 - SV_{40}	7136	—	2	1	1	NT
	7121	—	—	1	1	NT
" 20 - SV_{40}	7077	—	2	2	2	NT
CELO	5902	—	—	—	—	NT

* — = No effect. † NT = Not tested. ‡ P = Passage.

nated Huebner-Price). However, the tumor cells originally induced by the adenovirus 7-SV_{40} hybrid virus were shown to contain adenovirus tumor antigen, since hamsters carrying tumors produced by the tumor cells grown in tissue culture developed antibodies to both the SV_{40} and adenovirus tumor antigens(8). The 2 rat cell lines have not been successfully transplanted in rats, hence it was possible that they did contain antigen at levels which were not detectable by direct tests for antigen content. Thus, the calcium-positive cell lines that were tested adequately in every case were shown to contain virus-specific tumor antigens. A cell line derived from a hamster tumor(14) induced by CELO virus, an agent reported to be an avian adenovirus(15), was calcium-negative as well as negative for CF antigens(14). Calcium sensitivity, however, was not limited to tumor cells induced by human adenovirus since cells derived from tumors induced by a simian adenovirus (SV20) were calcium sensitive.

The individual adenovirus lines varied in their sensitivity to calcium. Some showed an effect after 24 hours in 2.0 mM calcium; others showed no effect until the fourth day of exposure to 7.5 mM calcium (Table II). However, each of 7 adenovirus lines carried for long periods reacted in the presence of 1.8 mM calcium, the concentration normally present in Earle's Balanced Salt Solution. The most sensitive line studied was Levinthal's adenovirus type 12-transformed rabbit kidney (13). This line had an optimal calcium concentration of 0.05-0.1 mM. At lower concentrations, a calcium deficiency developed after several passages and the cells detached from the glass; at higher concentrations, the cells demonstrated the typical calcium effect in 24 hours (Fig. 1).

Preliminary experiments indicated the clumping phenomenon was also influenced by variations in pH. Sensitivity to calcium increased as the pH was increased. However, when it was measured to the point which

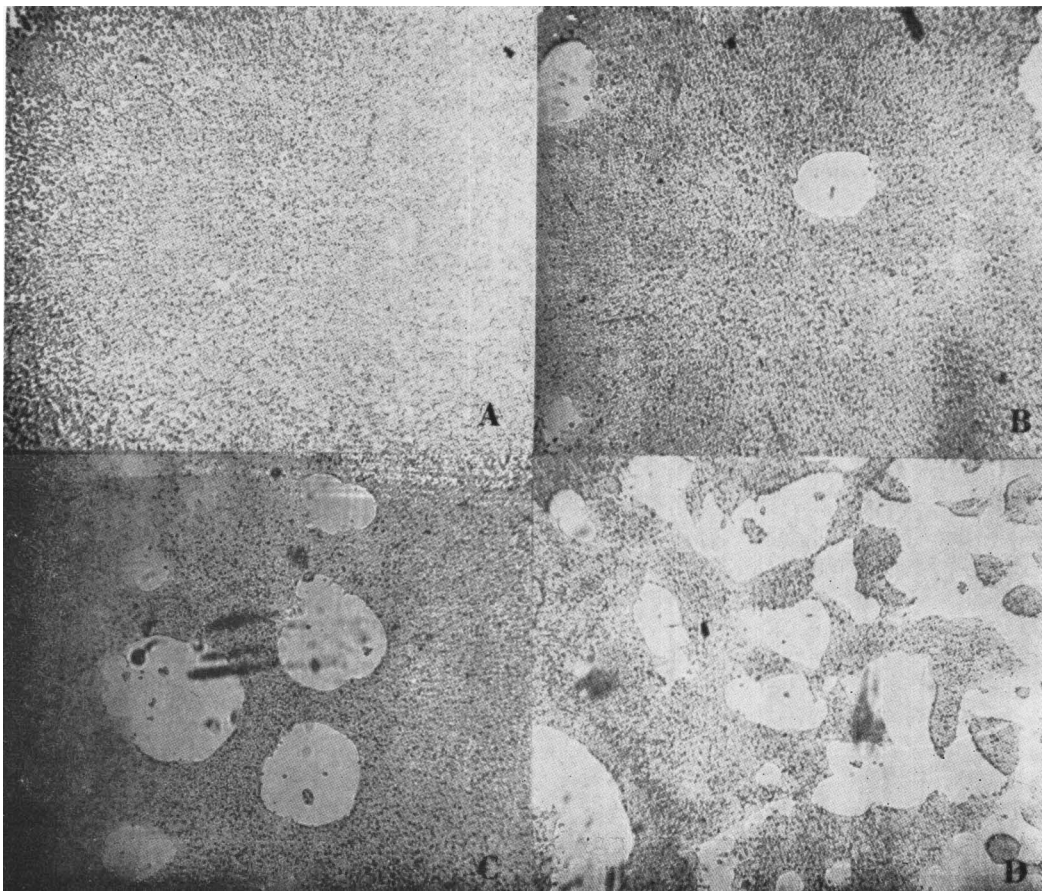

FIG. 1. Effect of calcium on adenovirus type 12-transformed rabbit kidney (Levinthal) after 18 hr exposure. × 43.75. (A) 0.1 mM Calcium, (B) 0.2 mM Calcium, (C) 0.4 mM Calcium, (D) 0.8 mM Calcium.

induced precipitation, the clumping effect of calcium salts was reversed. Conversely, cells were less calcium sensitive when the cultures were maintained at pH's below 7.

Discussion. In a previous publication(7) it was demonstrated that 2 cell lines derived from adenovirus type 12-induced hamster tumors were sensitive to 1.8 mM calcium after a number of passages. The first efforts to extend this study showed that the 7 cell lines tested (Table II) were calcium sensitive after 5-10 passages.

To study a number of cell lines in this way would have required a long and tedious effort. The present test, based on 7.5 mM calcium, was devised to reduce the time needed for detecting calcium sensitivity. This test indicated that there was a correlation between calcium sensitivity and cell lines derived from adenovirus-induced tumors or adenovirus-transformed cell lines, regardless of species of origin.

One would expect a normal distribution curve with most of the cell lines falling in a mid-range and only a few on the shoulders. It would not be surprising if a few of the more calcium sensitive non-adenovirus-induced cell lines should overlap with a few of the less sensitive adenovirus-induced cell lines, since the calcium effect itself is probably an exaggeration of some normal role of calcium in cell function. However, it is possible that many calcium sensitive non-adenovirus-induced cell lines have never been derived because of the calcium concentration in the media used for isolation.

The findings suggested several practical and/or theoretical implications:

Calcium sensitivity may be useful as a marker in recognizing adenovirus-induced tumors or -transformed cell lines. Such an additional marker may prove useful because (a) certain tumors arising subsequent to an inoculation with adenovirus may not be induced by the virus, and (b) it is known that certain cell lines derived from adenovirus-induced tumors contain insufficient tumor antigen to be detected by directly testing the cells for antigen content(16).

Serial passages of cell lines derived from adenovirus-induced tumors or adenovirus-transformed cell lines may be facilitated by using a medium containing a relatively low (0.1 mM) concentration of calcium. This concentration was found to be below the toxic level and above the minimum requirement level for each of the 26 adenovirus-induced or -transformed cell lines studied. Further studies, however, are necessary to determine if the use of this low calcium medium eventually alters oncogenic or serologic behavior of these cells.

Transformation *in vitro* by adenovirus of cultures derived from normal tissue may be difficult to demonstrate due to an unfavorable calcium environment for the newly transformed cells. Upon being transformed, these cells would begin to multiply and the newly formed clones would tend to form balls, detaching from the glass and being discarded with the removal of the spent medium. By using a low calcium medium, it may be possible to demonstrate transformation more readily.

Calcium sensitivity may reflect an alteration in cell metabolism which is mediated specifically by adenovirus during malignant transformation.

Summary. Cell lines derived from adenovirus-induced tumors or adenovirus-transformed cell lines clumped or retracted in 7.5 mM calcium or less, a characteristic generally not shown by cells derived from other virus-induced tumors or cells transformed by other viruses. Similarly, standard primary, diploid, and heteroploid cell cultures were not sensitive to 7.5 mM calcium. In support of these observations, the use of a low calcium medium facilitated the passaging of adenovirus-transformed cells in tissue culture; however, the use of a completely calcium-free medium resulted in a deficiency which caused detachment of the culture. The calcium effect may be useful as a marker to substantiate other evidence that a tumor was induced by adenovirus or that a cell line was transformed by adenovirus.

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